DIETARY MAGNESIUM INTAKE AND ATHEROSCLEROTIC PLAQUE DEVELOPMENT IN RABBITS

BY

JENNIFER LYN KING

B.S., University of Illinois at Urbana-Champaign, 1999

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CHAPTER 1: INTRODUCTION/LITERATURE REVIEW

Magnesium Physiology and Deficiency

The available literature on magnesium (Mg) was summed up by the 1997 Dietary Reference Intake (DRI) Committee: " The ability to determine reference dietary intakes for Mg is, as indicated throughout this chapter, hampered by available data" (1). They identified several areas that were in particular need of clarification and/or further study. These included reliable population intakes, reliable and specific biomarkers, basic studies in healthy individuals (including depletion studies to measure Mg pool changes), intervention studies, and toxicity of pharmacological doses of Mg.

Deficiency of Mg was first studied by McCollum and colleges at Johns Hopkins in the 1930s (2). Dogs and rats were fed a diet that provided a mere 1.8 ppm of Mg. They found that severe Mg deficiency led to vasodilation, hyperirritability, muscle disturbances, and fatal convulsions. It is best said in Kruse's own words: "This morbid condition, so rapid in its onset, so short and stormy in its course, and so fatal in its termination, leaves no doubt that Mg is an essential element for the animal body" (2). They found that the tetany from Mg deficiency is distinguishable from other causes of tetany because Mg deficiency caused no carpopedal (wrist or foot spasms) or laryngospasm (a spasmodic closure of the larynx) and accompanying it was a prominent vasodilation. They also found that there was a consistent lowering of blood calcium (Ca) that occurred along with a lowering of blood Mg. After 4 weeks of depletion in the dogs, there was a sudden rise in serum cholesterol which peaked and then plateued until death. Just before death, they also observed a decrease in erythrocytes and an increase in non-

protein nitrogen. There were 3 dogs in the study, one died at 7 weeks, the other 2 died at 17 weeks.

Many diverse clinical manifestations have been reported with Mg deficiency, including accelerated atherosclerosis, asthma, neurological and psychiatric clinical conditions, and sudden death (3). While severe Mg depletion has readily apparent cardiovascular (CV) and neurological symptoms, it is harder to identify early or marginal deficiency (4). Symptoms can include poor appetite, irritability, weakness, muscle tremor, tetany, twitching, numbness, tingling, disorientation, apathy, memory loss, and skin lesions (5). Diseases that have been linked to low Mg include cancer, kidney stones, arthritis, insomnia, menstrual cramps, and chronic fatigue syndrome (5).

Low dietary Mg intake has been demonstrated to result in hypertension, atherogenesis, and stroke (6). Diets high in Mg have been shown to decrease incidence of hypertension, atherogenesis, myocardial infarction, and stroke (6). In rats, dietary Mg deficiency not only caused hypertension, but also vascular remodeling (6). Patients with hypertension, ischemic heart disease, and stroke exhibit significant depletion of serum ionized Mg (6). In vitro studies with aortic and cerebrovascular smooth muscle cells show that reduction in Mg below the normal human, rat, and dog serum value of ~0.6 mmol/L induces early expression of at least two proto-oncogene and the transcription factor NFkB (6).

Inability to adequately form bone, as well as lack of Mg for bone mineralization may be among the most important outcomes of Mg deficiency in small children (4). Both diabetes and many diuretic drugs are known to compromise Mg status by promoting renal Mg loss (7). It has been shown that in a variety of patients, with ongoing atherosclerotic disease processes, including hypertension and stroke, exhibit significant depletion of

serum ionized Mg2+ 18-22 (6). Hair mineral analysis has been used to estimate the deficiency of minerals in the US population and using this technique Mg was found to be the 2nd most deficient mineral after chromium (Table 1) (5).

Mineral	Deficient (%)
Chromium	56
Magnesium	49
Zinc	47
Calcium	46
Manganese	40
Selenium	40
Potassium	37
Iron	25
Copper	25
Molybdenum	15
Phosphorus	9
Sodium	6

Table 1. Deficiency of minerals via hair mineral analysis (5).

Mg is used to treat cardiac arrhythmias, myocardial infarction, asthma, pre-eclampsia and eclampsia in clinical practice (8). The safety of pharmacological doses of Mg has not been assessed though. This could lead to incidences of toxicity. Signs of Mg toxicity are vomiting, hypotension, bradycardia, and other arrhythmias, somnolence, and weakness (9) although it should be noted that these have only been seen in patients receiving Mg intravenously. No cases of toxicity or adverse effects when Mg is consumed as a food

have been identified, which is why the 1997 Upper Limit (UL) for safety was chosen based on non-food sources (including antacids) at a level which caused an adverse reaction (namely diarrhea) (1).

The following can increase renal elimination of Mg, when consumed in excess: alcohol, diuretics, coffee, tea, salt, phosphoric acid, Ca, potassium (K) and sugar (10). Conditions that can also increase Mg elimination include prolonged diarrhea or vomiting, biliary fistulas, chronic pancreatic insufficiency, aldosteronism, diabetic ketoacidosis, prolonged intense stress, very heavy menstration, and sweating (10). Insufficient water intake, a very common phenomenon, has the same effect as high sodium intake, causing increased elimination of Mg, in order to increase the salinity of the urine (10).

The kidneys require Mg in order to recycle Ca, phosphorus (P) and K and to eliminate excess sodium (Na) and chloride (Cl). A Mg deficiency eventually leads to low serum levels of Ca and K and to high levels of Na and Cl. This electrolytic imbalance leads to hypertension and to intracellular disturbances (10). Mg is capable of forming soaps with fatty acids in the intestine and thus reducing the digestible energy content of the diet (11). Mg requires both PTH and vitamin D for absorption and a diet rich in saturated fats can hinder Mg absorption (10). Mg absorption has been estimated to be 59% (12). Absorption is mainly dose dependent, making comparison between studies difficult (12). The highest absorption is when Mg levels are low in the lumen of the intestines. However the higher absolute amount of Mg absorbed was obtained with the higher amount tested (12). Therefore, it is preferable to compare the absolute amount absorbed rather than the fraction absorbed. The short half-life and expense of ^{28}Mg radioisotope has hampered bioavailability studies (12).

Efficient insulin activity requires an optimal intracellular concentration of free Ca while undue increase in intracellular free Ca can compromise the ability of insulin to promote efficient glucose uptake (7). Mg works in a variety of ways to aid the control of intracellular free Ca. Extracellularly, it acts as a mild, non-specific antagonist of various Ca channels , moderating intracellular Ca uptake and it promotes Ca extrusion as well as sequestration of Ca in endoplasmic reticulum (7). Ca, P, and Mg have important intracellular and extracellular functions with their metabolism often linked through common hormonal signals (13). Extracellular Mg concentrations are largely controlled by the kidney with the renal tubular max reabsorption controlling the plasma concentration (13).

Mg modulates vasomotor tone, blood pressure, and peripheral blood flow (14). Mg is partially bound to proteins in complex with small anion ligands and as free ionized Mg (14). A small proportion of ionized Mg in the circulation is thought to exert several beneficial effects on vascular endothelial function (14) and measurement of serum Mg does not always reflect the overall status of Mg metabolism (14). Serum Mg correlates well with intracellular free Mg, the physiologically active form (14). While not the best indicator or total Mg status, serum Mg is the most practical and commonly used parameter for assessing disorders of Mg metabolism in clinical practice (14).

A number of physiological, nutritional, and biochemical control mechanisms are responsible for normal functions of the peripheral and cerebral vasculatures. These homeostatic factors maintain both the patency of the blood vessels and the fluid of the blood (15). Mg reduces arteriolar tone and tension in a wide variety of arteries (16). It has been shown that cAMP induces Mg release from mitochondria (17).

Mg is crucial for the function of G proteins which play important roles in mediating the intropic effects of β-adrenergic agonists in the heart and are altered in heart failure (17). Mg is required for protein and nucleic acid synthesis, maintenance of the cell cycle, cytoskeletal and mitochondrial integrity and for the binding of substrates to the plasma membrane (17). In a typical mammalian cell ~90% of total cellular Mg is bound to ATP in the cytosol or within nuclei, mitochondria, and endoplasmic reticulum (17).

Mg has been proposed to act as a chronic regulator of cell functions , opposed to Ca which is responsible for acute events (18). Mg directly antagonizes Ca and is a second messenger in angiotensin II signaling in smooth muscle cells. Mg deficiency interferes with endothelial production of nitric oxide, a vasodilator and inhibitor of platelet aggregation and adhesion, which is reduced in atherosclerosis (18). Mg levels influence the synthesis of nitric oxide, intracellular Ca release, the uptake and metabolism of LDL, the permeability to water and albumin, and the proliferation of endothelial cells (19).

Mg is involved in the conversion of essential dietary fatty acids to prostaglandins and prostacyclins which are potent vasodilators and platelet antiaggregators (20). It is an intracellular mineral required for the conversion of glucose to glucose-6-phosphate (5). The plasma Mg level is maintained remarkably constant in healthy individuals by poorly understood mechanisms (21).

In humans 30% of bone Mg content is in the surface-limited pool and the remaining 70% bone mg is integrated into the bone crystals (22). Mg is the fourth most abundant cation in the body and the second most abundant intracellular cation after K. The most significant proportion of cellular Mg is bound to various cell constituents and ionized Mg

is only a very small percentage of the total although it is the most important in that it is biologically active (22).

The measurement of free Mg posed a significant methodological problem until the synthesis of a new fluorescent Mg sensitive dye has recently allowed the measurement of intracellular free Mg in nucleated cells (23). Despite the important physiological roles of Mg and its proven or potential benefits, surveys show that the dietary intake of Mg is inadequate in the US (8).

Dietary Intake and Status of Magnesium

The known risk factors for CVD, including specific dietary components (e.g. excess saturated fat and cholesterol) explain only a portion of the morbidity and mortality due to CVD . It is now recognized that we must focus on the total diet and not just on individual dietary components. As a result, the study of patterns of intakes of nutrients, foods, and food groups has emerged in nutrition research. Because many nutrients are highly correlated with the same foods, it is difficult to examine their effects separately (24).

The key recommendations from the Dietary Guidelines for Americans 2005 are: consume a sufficient amount of fruits and vegetables while staying within energy needs; choose a variety of fruits and vegetables several times/week; consume 3 or more ounce-equivalents of whole-grain products /day and at least ½ grains should be whole grains; and consume 3 servings /day of fat-free or low-fat milk or milk products (25). All of these recomendations would increase Mg intake. Mg was listed as a nutrient of concern for adults and children in the 2005 Dietary Guidelines (25). Dietary intake of Mg is sub-

optimal with shortfalls of between 65 and 175 mg of Mg/d, depending on geographic regions (15). African Americans, the less educated, and the elderly, have particularly low intakes of Mg (8). It was found that 23% of US adults aged 25-74 years old had a serum Mg concentration <0.8 mmol/L, which is consistent with hypomagnesia (8). The median dietary intake of Mg is lower than either the EAR, or the RDA in all groups suggesting

Sex/Group	Age	RDA (mg/day)	UL * (mg/day)
Infants	0-6 months	30 (AI)	None
Infants	7-12 months	75 (AI)	None
Children	1-3 years	80	65
Children	4-8 years	130	110
Children	9-13 years	240	350
Boys	14-18 years	410	350
Men	19-70 years	400	350
Men	>70 years	420	350
Girls	14-18 years	360	350
Women	19-70 years	310	350
Women	> 70 years	320	350
Pregnancy		350-400	350
Lactation		310-360	350

Table 2. 1997 DRI suggested RDA values (1).
*upper limit (UL) is based on supplements

that Mg intake from dietary sources in the US remains suboptimal (Table 2) (1).

Several epidemiological studies found that diets rich in whole grains may protect against CVD, stoke, type-2 diabetes, and certain cancers (7,26). Whole grain intake has been shown to be inversely associated with waist to hip ratio, LDL cholesterol, and fasting insulin (26). The protective effects of whole grains may depend on the presence or interaction of several biologically active constituents, including dietary fiber, vitamin E, Mg, folate, and other nutrients and non-nutrients (26).

Mg is a major constituent of the germ and whole grains provide much higher levels of Mg than refined grains. Whole grains are one of the best dietary sources of Mg. Other foods which are equally rich in Mg (on per calorie basis) are nuts, legumes, and reduced fat dairy products (Figure 1 and Table 3) (7).

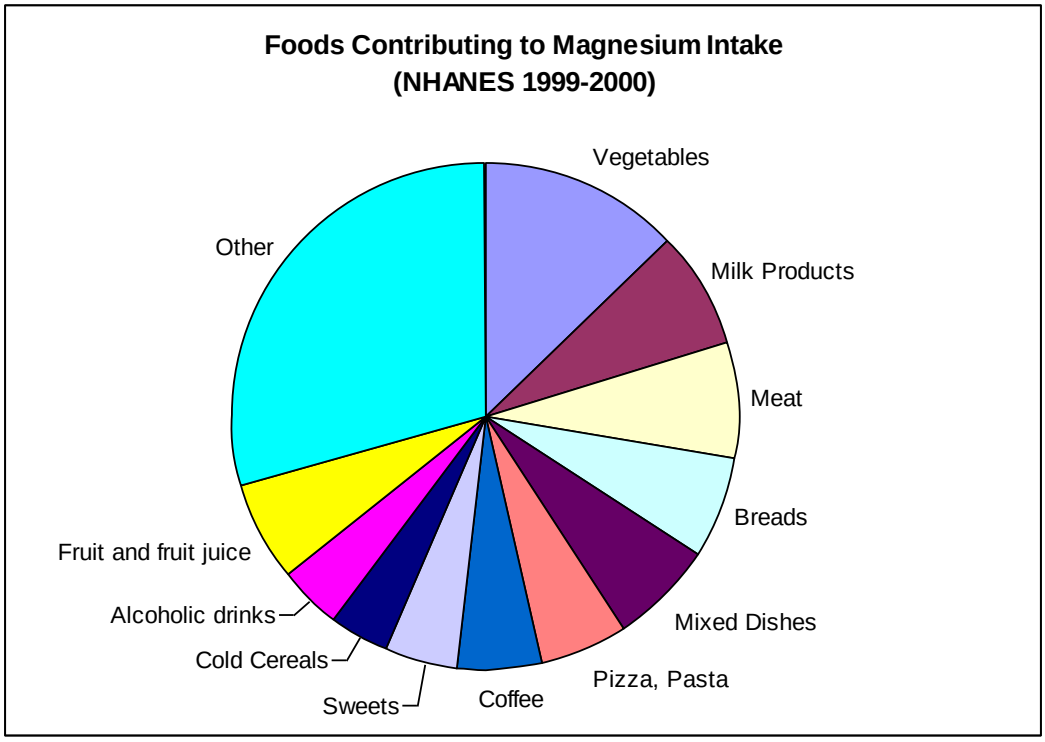


Figure 1. Foods that contribute to Mg intake (8).

Each of these foods have been linked to decreased risk of type-2 diabetes and CVD in epidemiological studies (7).

Food	Measure	mg Mg per measure	kcal	Mg density (mg of Mg/kcal)
Canned Spinach	1 cup	163	49	3.33
Kellog's All-Bran Cereal	1/2 cup	109	81	1.35
Pumpkin Seeds	1 oz	151	148	1.02
Halibut	1/2 fillet	170	223	0.76
Oat bran muffin	1 muffin	177	305	0.58
Brazil nuts	1 0z	107	186	0.58
Lima beans	1 cup	50	94	0.53
Black Beans	1 cup	120	227	0.53
Tomato paste	1 cup	110	215	0.51
Boiled Soybeans	1 cup	148	298	0.50
Almonds	1 oz	78	164	0.48
Trail Mix	1 cup	301	707	0.43
Whole-grain wheat flour	1 cup	166	407	0.41
Brown rice	1 cup	84	216	0.39
Whole-wheat bread	1 slice	24	69	0.35
Milk	1 cup	27	83	0.33
Yogurt	8 oz	39	143	0.27
Semisweet chocolate	1 cup	193	805	0.24
White bread	1 slice	6	66	0.09

Table 3. Dietary Sources of Mg (27)

Cardiovascular Disease and Magnesium

Atherosclerosis was first described by Lobstein in 1829 and since then, many theories have emerged on its pathogenesis and origin (28). The American Heart Association (AHA) reported in 2005 that coronary heart disease (CHD) was the single largest killer of Americans and that atherosclerosis was the cause of the majority of cases of CHD (29). Since the first animal experiments by Anitschkow in 1913, cholesterol deposition into the arterial wall has been recognized as the primary event in the process of atherosclerosis (28). The development of atherosclerosis involves a multitude of factors including dietary habits, glucose and insulin levels, age, sex, and blood lipids (30). There is a consensus that atherosclerosis is a complicated disease of the arterial wall arising from a chronic inflammation that is the result of lipo-proteins entering the vascular lumen of the artery (28,31). Lipid deposition itself is not the entire cause, rather the body's defense mechanisms against the lipid seems to be the culprit (28). Macrophage activation and smooth muscle transformation into fatty "foam" cells is necessary to form an atheroma (18). Mechanisms involving coagulation play a role in the initial and in the late stages (28). The production of fibrous tissue produces the classic, late stage atherosclerotic lesion which will lead to clinical events such as myocardial infarction (MI), stroke and other cerebral vascular diseases, and peripheral vascular disease (PVD) (28).

Traditional risk factors like age, sex, elevated blood pressure, smoking, elevated LDL, and depressed HDL have been clearly demonstrated to correlate with incidence of atherosclerosis (32). More than any single factor, the "global risk profile" or the sum of all risk factors, can provide better prediction of cardiovascular events (Table 4) (32). The US National Cholesterol Education Program has adopted a system of scoring risk, called

the Framingham risk point scores, which is based on the traditional risk factors of age, sex, total cholesterol, LDL cholesterol, HDL cholesterol, systolic blood pressure, and smoking (32). The more recent Prospective Cardiovascular Münster (PROCAM) scoring system involves 8 variables including age, LDL cholesterol, HDL cholesterol, smoking, systolic blood pressure, triglycerides, family history of premature MI, and type-2 diabetes (32). Promising new risk factors have been considered, including homocysteine, oxidized LDL, insulin levels, and C-reactive protein (CRP) and will continue to be studied.

Older Risk Factors	**Newer Risk Factors**
Sex (men>women)	Metabolic Syndrome
Age	Impaired insulin function/NIDDM
Family History	Apolipoprotein B and A-1
Total Cholesterol	Small, dense LDL
LDL (High)	Oxidized LDL
HDL (Low)	Antibodies against oxidized LDL
Hypertension	Lipoprotein (a)
Smoking	Homocysteine
Obesity	CRP

Table 4. Cardiovascular Risk Factors (32).

Numerous studies have reported significant depressions in Mg serum levels of persons with vascular diseases (14) and there is evidence supporting the hypothesis that

dietary Mg intake can lower blood pressure and reduce incidence of stroke. The ARIC (Atherosclerosis Risk in Communities) Study showed an inverse relationship between dietary Mg and serum Mg with the development of carotid atherosclerosis (14). The first NHANES study found that for the highest 3rd and 4th quartiles of serum Mg concentration the risk of dying from heart disease was 20-30% lower (20). Some studies have not found this correlation. Levels of serum Mg were found to not be associated with evident ischemic heart disease (33).

Mg supplementation (30mmol/d) was given to patients with CAD and an improvement in exercise tolerance, exercise-induced chest pain, and quality of life scores was seen (16). This difference was seen in patients that were receiving optimal medical care for their CAD (96% on aspirin, 66% on lipid-lowering medications, 48% on beta-receptor antagonist, and 55% on ACE inhibitors). During Mg loading testing, patients with CAD absorb more Mg (less loss in urine), suggesting that these patients were in a Mg deficient state (16). These findings would seem to indicate that Mg might be a good addition to therapy for CVD.

Cholesterol and Magnesium

A reduction in total and LDL cholesterol and an increase in HDL cholesterol is associated with a decrease in risk for future coronary events in CVD patients (26). When excess dietary cholesterol is ingested, the metabolic changes that occur are suppression of endogenous cholesterol synthesis and increased re-excretion of cholesterol back into the intestinal lumen (28). HDL cholesterol is a carrier for cholesterol that needs to be transported from peripheral tissues to the liver for excretion. This and cell sloughing are

the only mechanisms for decreasing the body pool of cholesterol. High levels of HDL have been observed to provide protection from the progression of atherosclerosis (28). Mg has been proposed to increase conversion of TG to HDL by increasing the activity of lipoprotein lipase (11). Mg is also necessary for lecithin cholesterol acyltransferase (LCAT) activity (34).

It has been reported that serum Mg levels are inversley correlated to cholesterol levels in humans (35). There is the possibility that Mg and lipoproteins may behave differently in healthy subjects and in those having chronic diseases such as type-2 diabetes or CVD (35). In Mg deficient rats, concentrations of HDL were lowered and concentrations of chylomicrons, VLDL, and LDL were elevated (17). Animal studies have suggested that Mg deficiency can accelerate and intensify lipid deposition and lesions and that Mg supplementation can slow the progression of and prevent atherosclerosis (17).

Endothelium, Inflammation, and Magnesium

The endothelium is a thin cell layer that regulates the exchange of many molecules between the blood and the arterial smooth muscle. This includes the exchange of water and the maintenance of vascular tone and coagulation (31). In early atherosclerosis, monocytes differentiate into macrophages in the endothelium. This results in expression of scavenger receptors that ingest modified lipids, thus forming foam cells, the earliest atherosclerotic precursor (18). At this point, the damage is reversible, but progression of the atheroma into a fibrous plaque eventually leads to symptomatic, clinical disease (18).

Endothelial cells express many types of molecules, including adhesion molecules that attract monocytes to adhere (when injured), initiating the first step in atherosclerosis (31).

Activated immune cells are found in the arterial wall of the affected vessel, but also in other arteries (36). This tends to suggest a systemic inflammatory trigger that affects the endothelium in multiple areas and that antigens may be present in multiple vessels and even the myocardium itself (36). During dysfunction of the endothelium many tissue factors are expressed. Adhesion molecules are stimulated by interleukin-6 and other circulating cytokines and inflammatory molecules (31).

Mg deficiency has been shown to enhance endothelial injury and trigger vasoconstriction (14). Low serum levels of Mg have been shown to accelerate atherogenesis through promotion of inflammation and oxidative modification (14). Mg deficiency can promote the peroxidation of lipids and reduce antioxidant capacity in serum and tissues (37). Dietary Mg restriction has been shown to increase serum TG, stimulate lipid peroxidation, and increase lipid deposition into the vascular wall in rodents (14). Low serum Mg levels promote endothelial dysfunction, partially due to an up-regulation of inflammatory cytokines (38). Mg supplementation improved brachial artery endothelial function in patients with CAD and the effect of oral supplementation of Mg has been shown to be similar to the effects of lipid-lowering studies (16).

Thrombomodulin and Magnesium

The endothelium obviously plays a central role in vascular homeostasis and vascular diseases such as atherosclerosis. Biological markers of endothelial function would be a great asset for the prediction, evolution, and prognosis of cardiovascular disease, including the measurement of drug efficacy (31). Activation by circulating pro-inflammatory cytokines such as IL-6 and TNF-α cause the endothelium to express

adhesion molecules such as ICAM-1, VCAM-1, and E-selectin. This facilitates the migration and adherence of inflammatory cells to the endothelium (31).

The endothelial cells then release molecules that can be anti-coagulant or pro-coagulant in nature. An issue of marker measurement is that biological markers must be restricted to expression from the endothelium only. Candidate markers include E-selectin, von Willebrand Factor(vWF), soluble thrombomodulin (sTM), ICAM-1, and VCAM-1. Of these factors, von Willebrand factor and sTM are strongly increased during endothelial damage (31). Thrombomodulin (TM) is a molecule that has a role in the protein C anticoagulant pathway. It is expressed specifically by endothelial cells and can be released from endothelial cells upon injury as sTM (31). sTM has been demonstrated to be a specific marker of endothelial cell damage (29) and some studies have found high concentrations of sTM in arterial disease (39). Following endothelial injury by ultrasound, elevation of sTM was seen to be rapid and proportional to the severity of the injury (29). In a paper still in press, New Zealand White rabbits were fed a 1% cholesterol diet and sTM was seen to increase at 10 weeks, reached peak values at 14 weeks, and a significant correlation was found between sTM and LDL levels (39).

C-reactive Protein and Magnesium

C-Reactive protein (CRP) is a serum protein that has been associated with inflammation and increased risk of CVD events (38). CRP has been shown in vitro to reduce mRNA levels of TM in human endothelial cells (40). Low grade inflammation and elevation of CRP has also been associated with obesity, high blood pressure and diabetes (38). CRP seems to be an independent risk factor for cardiovascular events and

probably contributes to the disease process (41). Upon admission to the hospital ~70% of patients with severe unstable angina had elevated levels of CRP and proinflammitory cytokines (36). The AHA has recommended that further research be done on inflammatory factors and they have published guidelines that define risk levels for CRP (38). CRP seems to be an active mediator of atherogenesis as well as a predictor or CVD (40). CRP can directly affect endothelial cells, smooth muscle cells, and has been shown to stimulate uptake of LDL into macrophages (40).

Dietary Mg intake has been inversely correlated to CRP levels and decreased serum Mg levels have been documented in obese patients with elevated CRP (38). Those who consume less Mg were more likely to have elevated CRP and individuals in any quartile below the RDA were significantly more likely to have elevated CRP levels (38). Ways in which Mg status may affect CRP levels is not known at this time but it has been speculated that it is related to oxidative stress or endothelial dysfunction (38).

Animal Models for Atherosclerotic Research

The most widely used animal in atherosclerosis research is the rabbit (42). Nikolaj Nikolajewitsch Anitschkow was the first to observe the feeding cholesterol to rabbits induced atherogenesis, while feeding it to rats did not (43). The cholesterol concentrations in rabbits are very low when fed a normal chow diet (~50 mg/dL) but rise dose dependently when feed cholesterol. Feeding at least 0.5% cholesterol causes changes to the arterial intima which are comparable to early human lesions (43). The reason that rabbits are prone to atherosclerosis through cholesterol feeding is that they cannot increase the rate of excretion of sterols and as a result, the liver produces large

quantities of lipoproteins that are atherogenic, namely LDL and β-VLDL (42). These atherogenic lipoproteins remain in the blood for an extended period of time.

Dietary restriction of Mg, comparable to daily intakes by Americans, has been shown to exacerbate atherosclerosis in rabbits (18). Mg deficiency in high cholesterol fed rabbits has been shown to increase lipid deposition in the intima, leading to thickening of the intima (18). Feeding saturated fat to rabbits and supplementing with Mg led to a slight, but significant, decrease in atherosclerotic lesions in the aortic intima and in the total cholesterol content of the aorta (44). Pathology studies of these lesions showed many foam cells with large amounts of lipids.

Another study in which rabbits were fed 1% cholesterol diets with supplemented Mg showed no change in levels of cholesterol in the serum but aortic lesions and cholesterol content of the aorta were both decreased (44). The authors concluded that supplemental Mg inhibited lipid accumulation in the aorta wall. These findings support the epidemiological observations that Mg intake can help to decrease incidence of atherosclerotic disease.

Hypothesis/Goals

The goal of this study was to determine if supplemental levels of Mg (0.40%, High Mg group) would suppress and low levels of Mg (0.11%, Low Mg group) would increase atherosclerotic plaque development in rabbits, compared to a normal level of intake of Mg in the diet (0.25%, Normal Mg group). I hypothesize that atherosclerotic plaque, serum cholesterol, CRP, and sTM levels would inversely correlate to dietary Mg in a dose-dependent manner (Low Mg > Normal Mg > High Mg).

CHAPTER 2: MATERIALS AND METHODS

Preliminary Trials

Pilot trials were carried out to establish appropriate dietary levels of fat and cholesterol that would produce atherosclerotic lesions in rabbits with minimal adverse effects. Rabbits are prone to poor food intake (anorexia), fatty liver, and jaundice when fed diets high in fat (45). Several dietary levels of Mg were also evaluated in these pilot trials.

Four-month-old rabbits were fed a diet consisting of 20% fat and 2% cholesterol and containing either 0.11% Mg, 0.25% Mg, or 0.40% Mg for up to 7 weeks. Serum total cholesterol levels ranged from 736 mg/dL to 3255 mg/dL. Plaque was observed in all diet groups but the rabbits had difficulty with this high level of fat and cholesterol. Over time many rabbits incurred complications including anorexia and jaundice.

To decrease incidence of jaundice and anorexia another trial was run in which four-month-old rabbits were fed a diet consisting of 10% fat and 1% cholesterol containing 0.11% Mg for up to 11 weeks. Plaque was seen in the aortas after 6 weeks and there were fewer complications from this diet including a lower incidence of anorexia and no incidences of jaundice. A 10% fat and 1% cholesterol diet was chosen as the final diet because plaque was still observed, yet adverse reactions were minimized.

Animal Trial

Four-month old male New Zealand White rabbits (n=22) were acquired from Myrtle's Rabbitry, Thompson's Station, TN. After an acclimation period of one week on

standard chow (Catalog # 2031 Harlan Teklad, Indianapolis, IN), rabbits were weaned onto a semi-purified atherogenic diet over 10 days by substituting 10% daily increments of cholesterol diet for the standard rabbit chow. The basal test diet (Catalog # 1811279, TestDiet, Richmond, IN) contained 10% fat, 1% cholesterol, and 0.11% Mg and Mg oxide was then added to the basal diet to a obtain levels of 0.25% Mg and 0.40% Mg (Table 5). To assure quality of feed, diet samples were analyzed by an independent laboratory for Mg, fiber, and fat content (Waters Agricultural Laboratories, Inc., Camilla, Georgia). Rabbits were randomly assigned to one of the three diets, given deionized water, and fed *ad libitum* for 8 weeks. Feed intake was measured daily and rabbits were weighed weekly. Animals were housed individually in standard caging with stainless steel mesh bottom at normal temperature and light cycles. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign.

	0.11% Mg (Basal Diet)	**0.25% Mg**	**0.40% Mg**	**Harlan Teklad #2031 (Chow Diet)**
Fat (%)	10	10	10	3
Cholesterol (%)	1	1	1	0
Protein (%)	15	15	15	14
Fiber (%)	21	21	21	21
Magnesium (%)	0.11	0.25	0.40	0.19

Table 5. *Diet Composition*

At 8 weeks, rabbits were anesthetized with a mixture of ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg) administered subcutaneously. They were then euthanized with carbon dioxide. Aortas (aortic arch + abdominal aorta) were harvested and fixed in formalin. Heart, liver, and skeletal muscle were harvested and frozen at -70°C until analysis.

Serum Analysis

Blood samples were obtained at baseline, 2, 4, 6, and 8 weeks via the lateral or medial saphenous vein and placed into a non-coated tube (for serum) and a lithium heparin tube (for blood cell collection). Serum samples were allowed to sit for 1 hour at room temperature to promote clotting. They were then centrifuged at 3000 rpm for 10 minutes and serum was collected, aliqoted, and frozen at -70°C. Lithium heparin samples were washed with 0.9% saline and centrifuged at 1000 rpm for 10 minutes and supernatant was discarded. This was repeated twice (until supernatant was colorless). The cellular fraction left was then frozen at -70°C until analysis.

Control serum (Catalog # 410-00100, Wako Chemicals USA, Inc. Richmond, VA) was used in all cholesterol assays for quality control. Total serum cholesterol was measured using an enzymatic colorimetric kit (Catalog # 439-17501, Wako Chemicals USA, Inc. Richmond, VA). All samples except for baseline were diluted by a factor of 10 (1 part serum: 9 parts deionized water) because the levels in the cholesterol fed rabbit serum were beyond the usual standard curve of the kits. Triglycerides were measured using an enzymatic colorimetric method (Catalog # 997-37492 and #993-37592, Wako Chemicals USA, Inc. Richmond, VA). HDL was measured using a precipitation reaction

followed by an enzymatic colorimetric kit (Catalog # 431-52501, Wako Chemicals USA, Inc. Richmond, VA). LDL was then calculated using the equation: LDL=Total Cholesterol – HDL . Serum thrombomodulin was measured using an ELISA kit (Catalog #837, American Diagnostica Inc., Stanford, CT). Serum CRP was measured using an enzymatic colorimetric kit (Catalog #999-27001, Wako Chemicals USA, Inc.). Serum Mg was measured by the Clinical Diagnostic Laboratory at the College of Veterinary Medicine at the University of Illinois Urbana-Champaign.

Tissue Analysis

Liver lipids were extracted using a modification of the Folch method (46). The sample was placed in chloroform: methanol (1:1), homogenized, and filtered via gravity filtration. NaCl solution (0.29%) was added, vortexed briefly, and then centrifuged. The top layer was discarded and the interface was washed with 0.29% NaCl solution. The remaining solution was placed in a weighed test tube, evaporated, placed in a dessicator for at least 48 hours, and weighed to determine total lipids. Five ml of PBS was added to dried liver lipids, samples were resuspended at 37°C, and total serum cholesterol was measured using an enzymatic colorimetric kit (Catalog # 439-17501, Wako Chemicals USA).

Aortas were scored blindly in duplicate by a pathologist and a researcher at the Department of Pathobiology, College of Veterinary Medicine, University of Illinois Urbana-Champaign. A score of 0-5 was assigned to each aorta, with 0 being no plaque and 5 being the most plaque seen. After fixation in 10% buffered formalin/sucrose for a minimum of 24 hours, a parasagital section of the aortic arch was trimmed, embedded in

paraffin, sectioned at 3 μm, stained with hematoxylin and eosin (H&E) and mounted on glass slides for histological evaluation. Intima thickness was then measured in triplicate at the thickest point.

Statistics

Analysis of varience (ANOVA) statistics were employed using the SAS computer program (SAS Institute, Inc., Cary, NC) and Fisher's LSD method was used to determine statistical differences ($p \leq 0.05$) between diet groups and between time-points.

CHAPTER 3: RESULTS

Body Weight

There were no significant differences in body weights (BW) between groups at any time-point. The high Mg group's BW did not differ from baseline at any time point .

Diet	Week							
	1	2	3	4	5	6	7	8
Low	3.8 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	3.6 ± 0.1*	3.6 ± 0.1*
Normal	3.8 ± 0.1	3.7 ± 0.1	3.8 ± 0.2	3.6 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	3.5 ± 0.1*	3.3 ± 0.2*
High	4.0 ± 0.0	3.9 ± 0.1	3.9 ± 0.1	3.9 ± 0.2	3.9 ± 0.2	3.8 ± 0.1	3.6 ± 0.1	3.7 ± 0.1

Table 6. *Body Weights (kg)*
Mean ± SEM
** = significantly different from baseline ($p \leq 0.05$)*

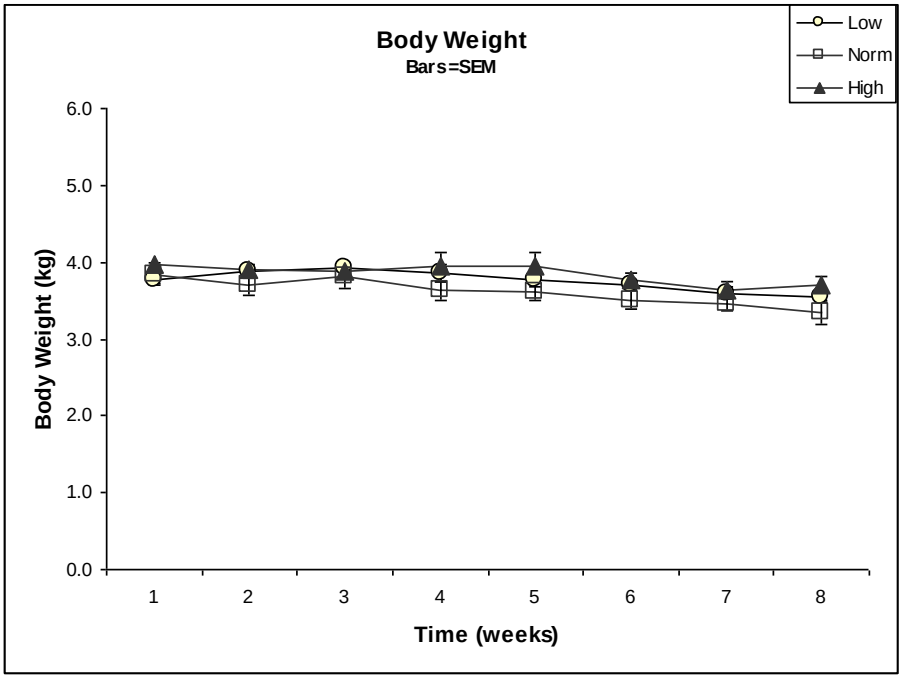


Figure 2. *Body Weight (kg)*
Mean ± SEM

The low Mg and normal Mg group's BW were slightly but significantly ($p \leq 0.05$) less than their baseline BW at 7 and 8 weeks.

Feed Intake

There were significant differences ($p \leq 0.05$) in feed intake at days 15, 17, 18, 19, 21, 34, 38, 39, and 42 (Table 7A, Table 7B, Figure 3). At days 15, 17, 18, 19, 21, 34, and 42 the high Mg group had a feed intake that was significantly ($p \leq 0.05$) higher than the low Mg group. The normal Mg group did not differ from either the high or low Mg group on those days. On day 38, the high Mg group had a higher feed intake than the normal group, and the low Mg group did not differ from either the high or normal Mg groups. On day 39, the high and low Mg group had no statistical difference in feed intake and both groups had a higher feed intake than the normal Mg group. When average daily feed intake for the entire study was calculated, the high Mg

Diet	Day							
	1	2	3	4	5	6	7	8
Low	132 ± 18	128 ± 26	92 ± 27	135 ± 50	69 ± 19	70 ± 17	77 ± 16	97 ± 22
Normal	132 ± 13	102 ± 15	99 ± 17	85 ± 21	60 ± 21	71 ± 23	52 ± 9	70 ± 20
High	160 ± 15	121 ± 22	125 ± 24	124 ± 25	100 ± 25	107 ± 25	103 ± 25	81 ± 21
Diet	Day							
	9	10	11	12	13	14	15	16
Low	85 ± 22	67 ± 18	74 ± 16	69 ± 16	73 ± 15	75 ± 13	55 ± 13 [b]	65 ± 8
Normal	63 ± 21	53 ± 21	52 ± 14	68 ± 14	66 ± 14	73 ± 21	80 ± 15 [ab]	74 ± 12
High	75 ± 20	73 ± 14	63 ± 7	63 ± 8	62 ± 8	80 ± 10	101 ± 14 [a]	96 ± 13

Table 7A. *Feed Intake (g)*
Mean ± SEM
Letters indicate significant difference between treatments ($p \leq 0.05$)

Diet	Day							
	17	18	19	20	21	22	23	24
Low	68 ± 9 [b]	66 ± 10 [b]	54 ± 14 [b]	59 ± 13	55 ± 8 [b]	66 ± 9	57 ± 13	53 ± 13
Normal	71 ± 13 [ab]	63 ± 15 [ab]	75 ± 15 [ab]	68 ± 16	68 ± 17 [ab]	65 ± 16	65 ± 13	58 ± 11
High	107 ± 14 [a]	102 ± 11 [a]	104 ± 11 [a]	102 ± 15	104 ± 15 [a]	89 ± 12	84 ± 7	81 ± 5
Diet	Day							
	25	26	27	28	29	30	31	32
Low	63 ± 12	74 ± 13	53 ± 9	58 ± 5	59 ± 5	55 ± 6	61 ± 4	65 ± 7
Normal	55 ± 15	62 ± 16	44 ± 13	71 ± 14	52 ± 14	58 ± 13	49 ± 13	50 ± 10
High	69 ± 5	74 ± 5	65 ± 8	74 ± 12	78 ± 10	75 ± 10	70 ± 7	68 ± 6
Diet	Day							
	33	34	35	36	37	38	39	40
Low	68 ± 11	40 ± 10 [b]	52 ± 6	55 ± 8	49 ± 7	45 ± 8 [ab]	53 ± 8 [a]	54 ± 9
Normal	56 ± 9	50 ± 11 [ab]	63 ± 8	80 ± 4	40 ± 6	34 ± 7 [b]	32 ± 3 [b]	53 ± 8
High	73 ± 7	75 ± 7 [a]	78 ± 14	59 ± 14	62 ± 10	62 ± 7 [a]	55 ± 5 [a]	60 ± 3
Diet	Day							
	41	42	43	44	45	46	47	48
Low	56 ± 9	53 ± 12 [b]	47 ± 11	52 ± 11	53 ± 7	55 ± 7	50 ± 8	52 ± 7
Normal	49 ± 7	59 ± 6 [ab]	86 ± 7	41 ± 6	41 ± 9	42 ± 9	43 ± 13	40 ± 12
High	64 ± 9	84 ± 11 [a]	65 ± 18	57 ± 7	52 ± 5	45 ± 5	55 ± 10	53 ± 9
Diet	Day							
	49	50	51	52	53	54	55	Avg Daily
Low	56 ± 6	51 ± 7	54 ± 9	44 ± 7	43 ± 9	44 ± 9	49 ± 9	64 ± 5[ab]
Normal	57 ± 10	57 ± 10	39 ± 10	28 ± 10	30 ± 13	26 ± 13	25 ± 13	59 ± 6[b]
High	69 ± 14	54 ± 14	47 ± 10	48 ± 10	55 ± 5	50 ± 6	46 ± 6	77 ± 6[a]

Table 7B. *Feed Intake (g)*
Mean ± SEM
Letters indicate significant difference between treatments ($p \leq 0.05$)

group had a significantly greater feed intake than the normal Mg group and the low Mg group was not statistically different from either normal or high Mg group.

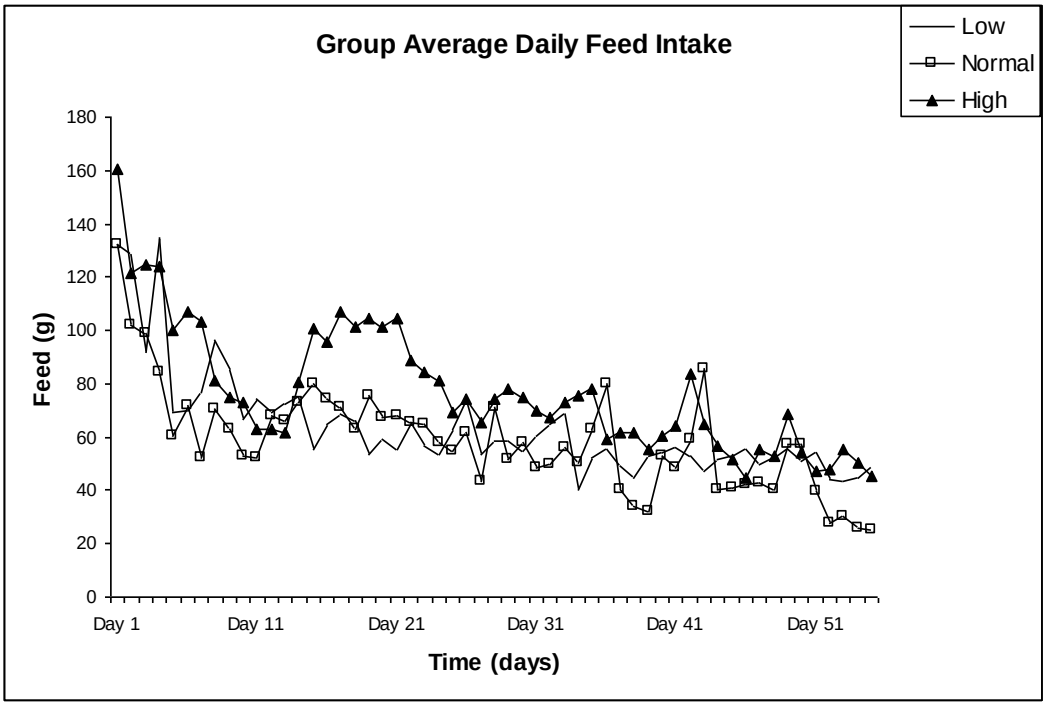


Figure 3. *Feed Intake (g)*
Mean ± SEM

Tissue Analysis

Liver weight (as % of body weight), liver lipids (as % of liver), liver cholesterol concentration (mg cholesterol/g liver), and total cholesterol in the liver (g) did not significantly differ according to dietary treatment (Table 9).

Liver Weight (% of Body Weight)			Liver Lipids (% of Liver)		
Low	Normal	High	Low	Normal	High
3.5% ± 0.3%	3.9% ± 0.1%	4.1% ± 0.3%	15% ± 2%	14% ± 1%	14% ± 1%
Liver Cholesterol (mg/g Liver)			Total Cholesterol in Liver (g)		
Low	Normal	High	Low	Normal	High
211 ± 13	190 ± 14	169 ± 23	24.6 ± 2.4	25.0 ± 7.0	26.0 ± 3.9

Table 9. *Liver Lipids and Cholesterol*
Mean ± SEM

Aortas from animals in the Low Mg group had more plaque in the aorta and aortic arch (Table 8, Figure 4, Picture 1-22) than the Normal or High Mg groups ($p \leq 0.05$). Amount of plaque present in the Normal and High Mg groups did not differ significantly. Histological examination of the aorta found similar results. The Low Mg group had a greater intimal thickness than the Normal or High Mg groups ($p \leq 0.05$) and the Normal and High Mg groups were not statistically different.

Diet	Intima Thickness (microns)	Score
Low	474 ± 58^{a}	3.4 ± 0.4^{a}
Normal	274 ± 49^{b}	1.6 ± 0.2^{b}
High	303 ± 58^{b}	2.0 ± 0.3^{b}

Table 8. *Intima Thickness and Aortic Plaque Severity Score*
Mean ± SEM
Letters indicate significant difference between treatments ($p \leq 0.05$)

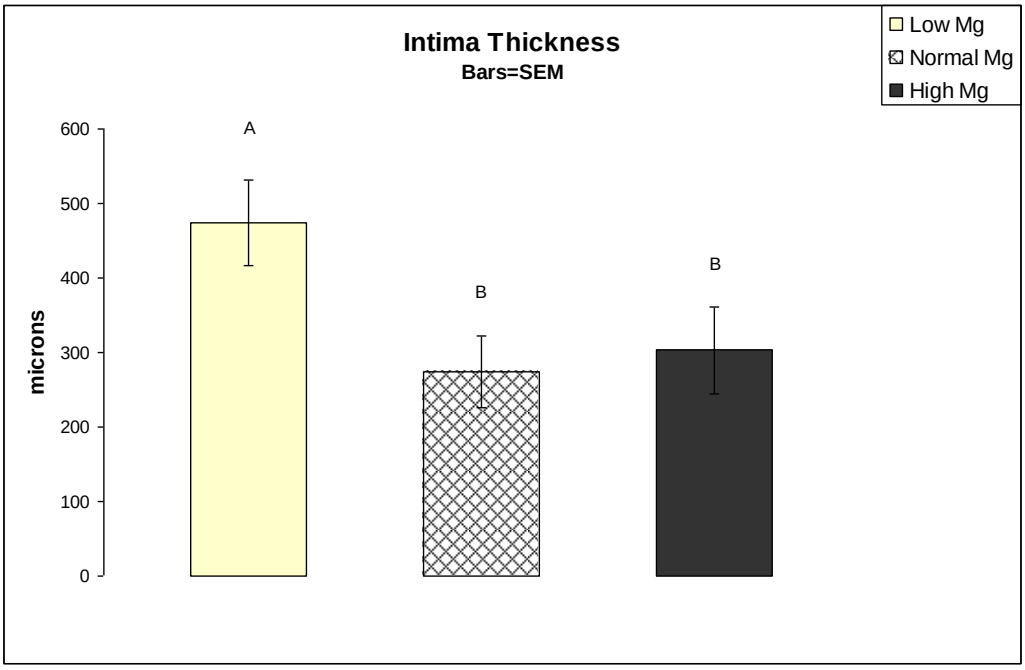


Figure 4. *Intima Thickness*
Mean ± SEM
Letters indicate significant difference between treatments ($p \leq 0.05$)

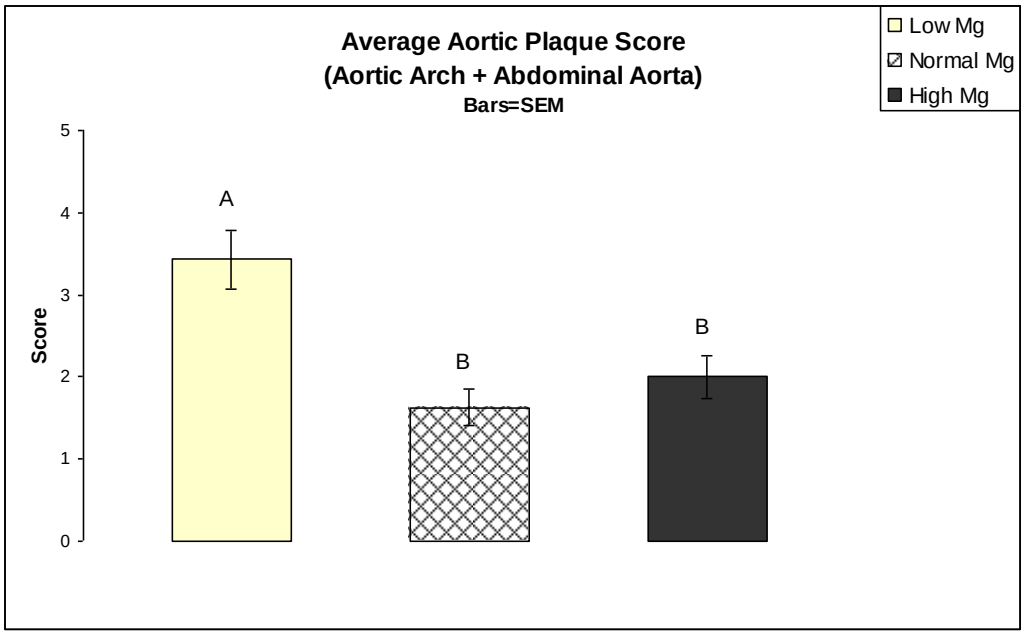
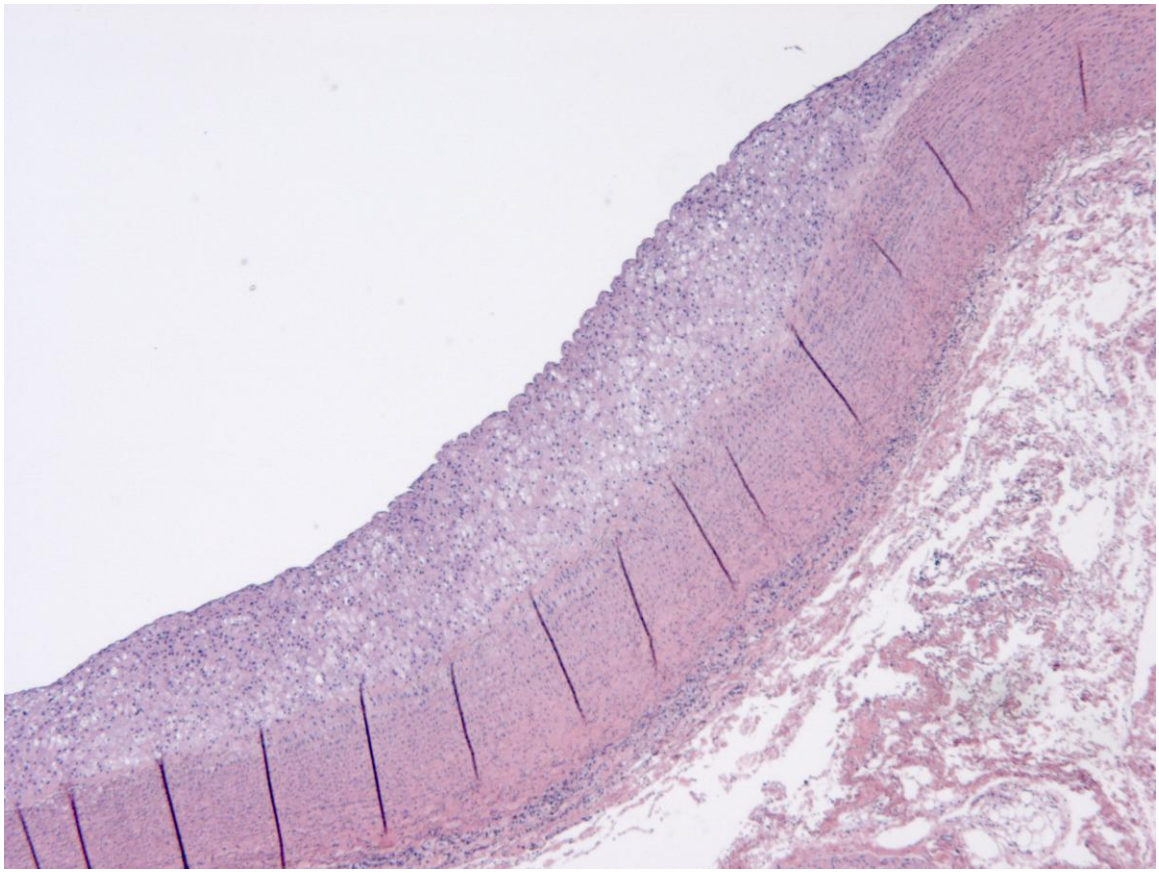


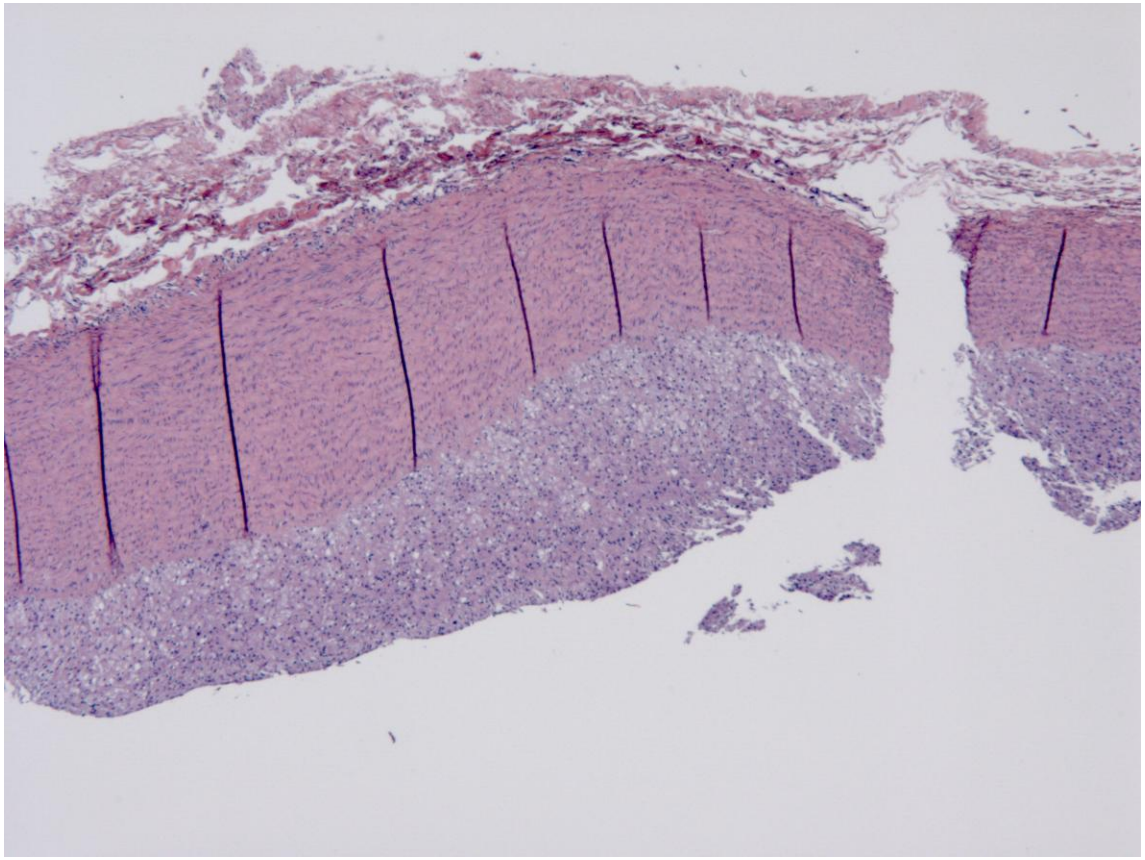
Figure 4. *Aortic Plaque Score*
Mean ± SEM
Letters indicate significant difference between treatments ($p \leq 0.05$)

Picture 1A. *Aortic Arch and Abdominal Aorta (L428 Low Mg)*

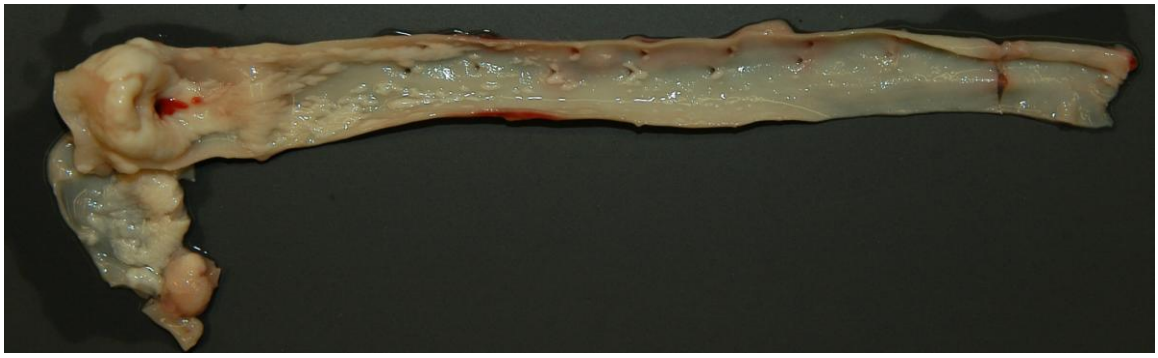


Picture 1B. *H&E Stain of Aorta* (*L428 Low Mg)*

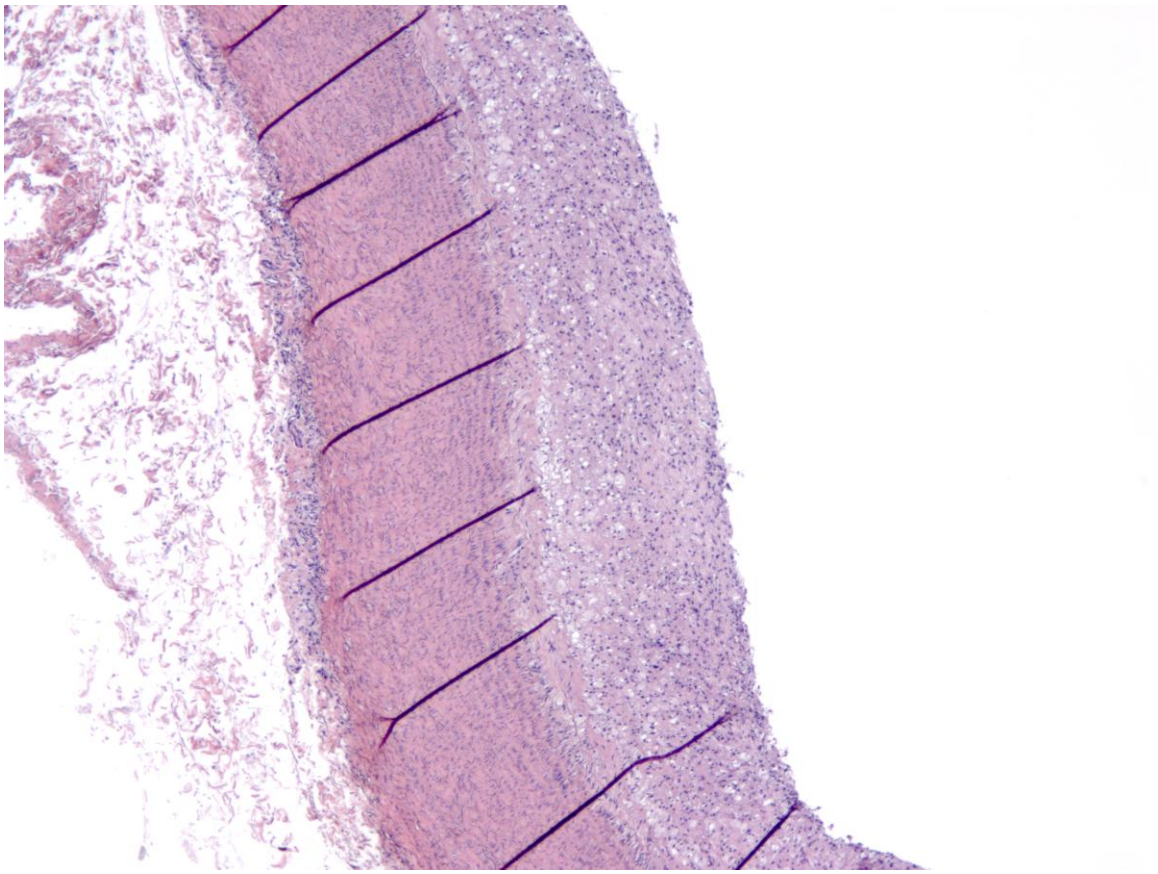
Picture 2A. *Aortic Arch and Abdominal Aorta (L431 Low Mg)*



Picture 2B. *H&E Stain of Aorta* (*L431 Low Mg)*

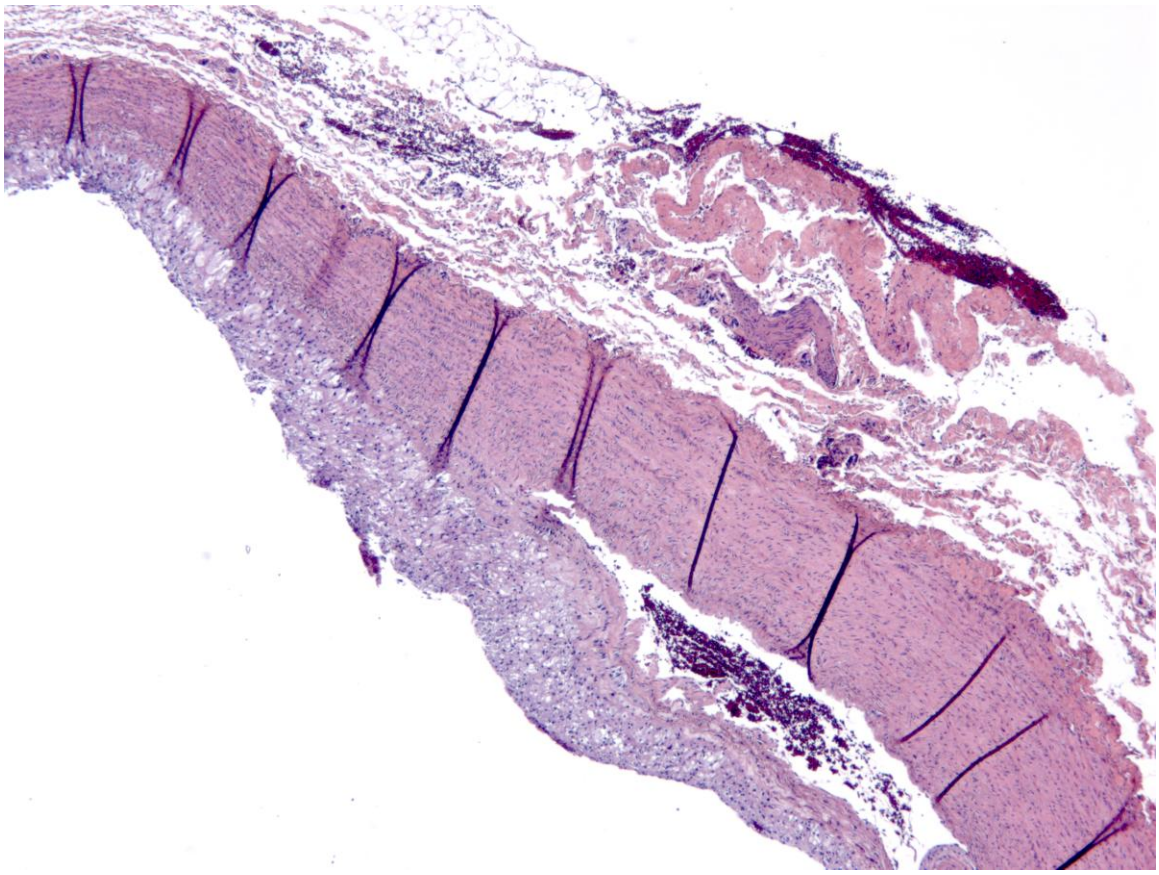


Picture 3A. *Aortic Arch and Abdominal Aorta (L437 Low Mg)*



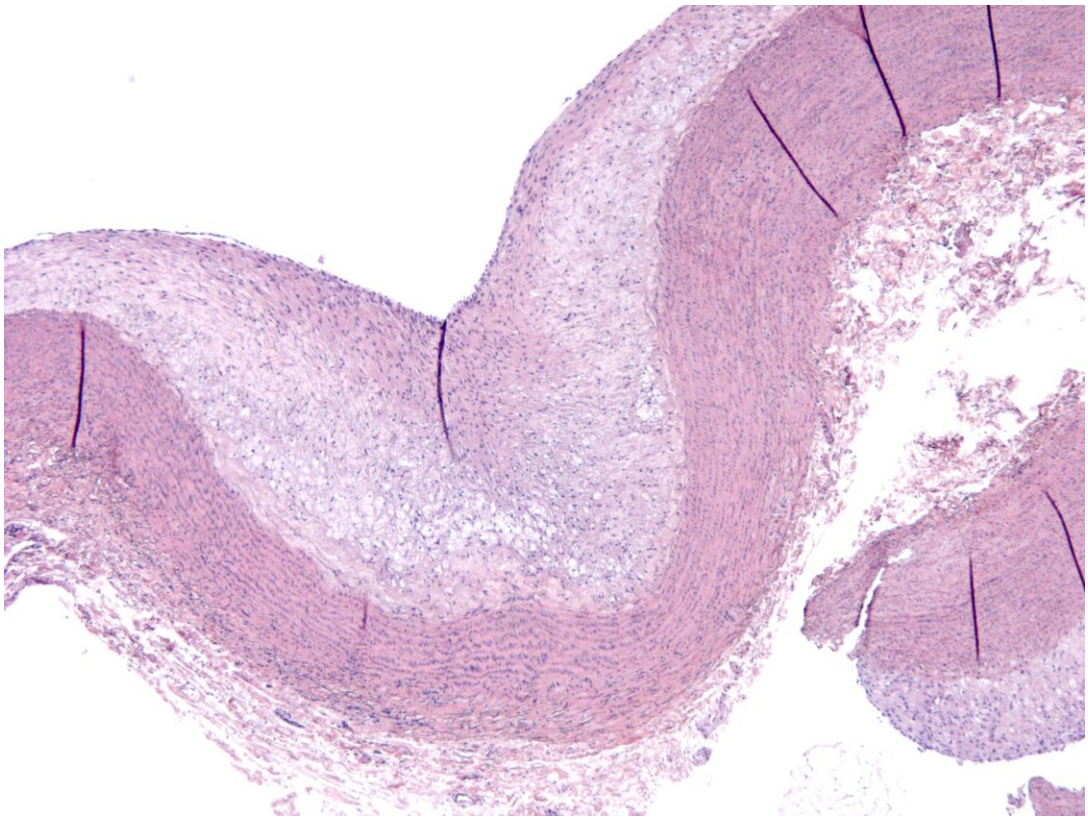
Picture 3B. *H&E Stain of Aorta* (*L437 Low Mg)*

Picture 4A. *Aortic Arch and Abdominal Aorta (L446 Low Mg)*



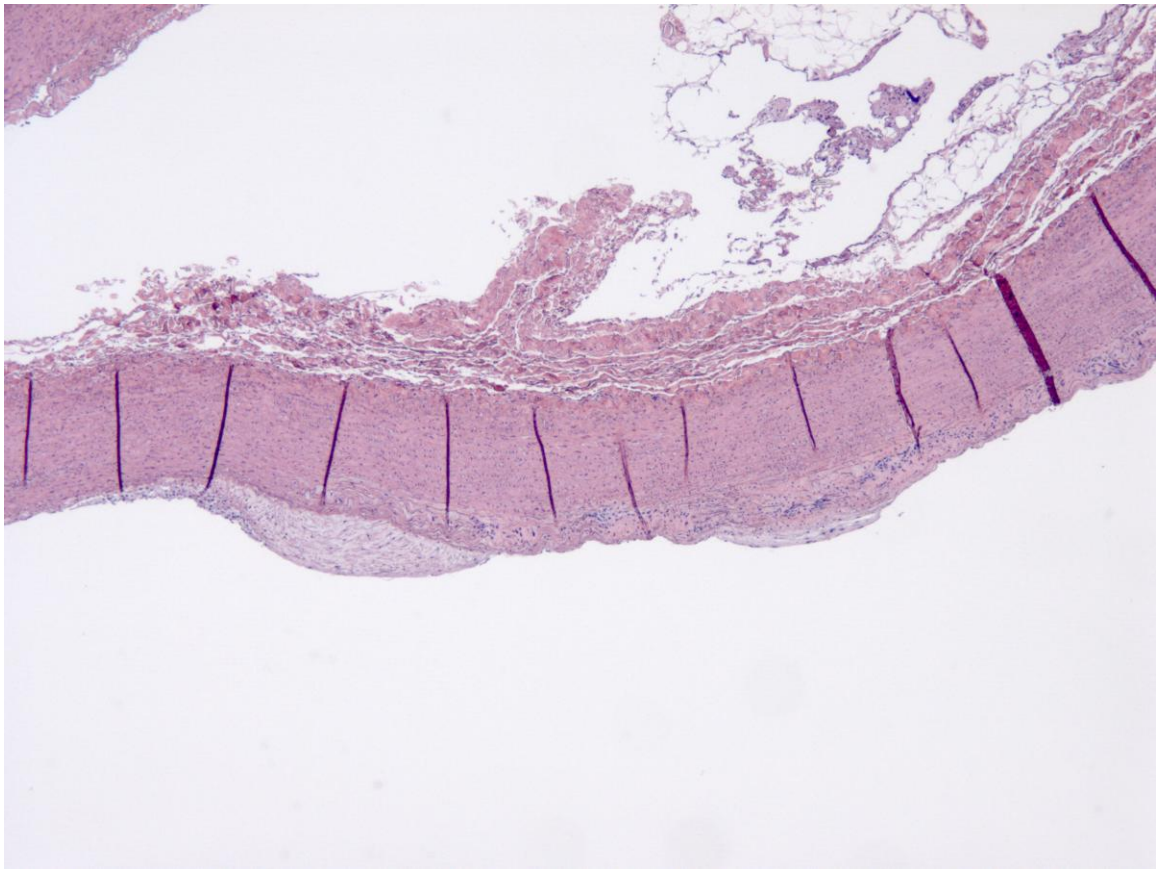
Picture 4B. *H&E Stain of Aorta* (*L446 Low Mg)*

Picture 5A. *Aortic Arch and Abdominal Aorta (L449 Low Mg)*



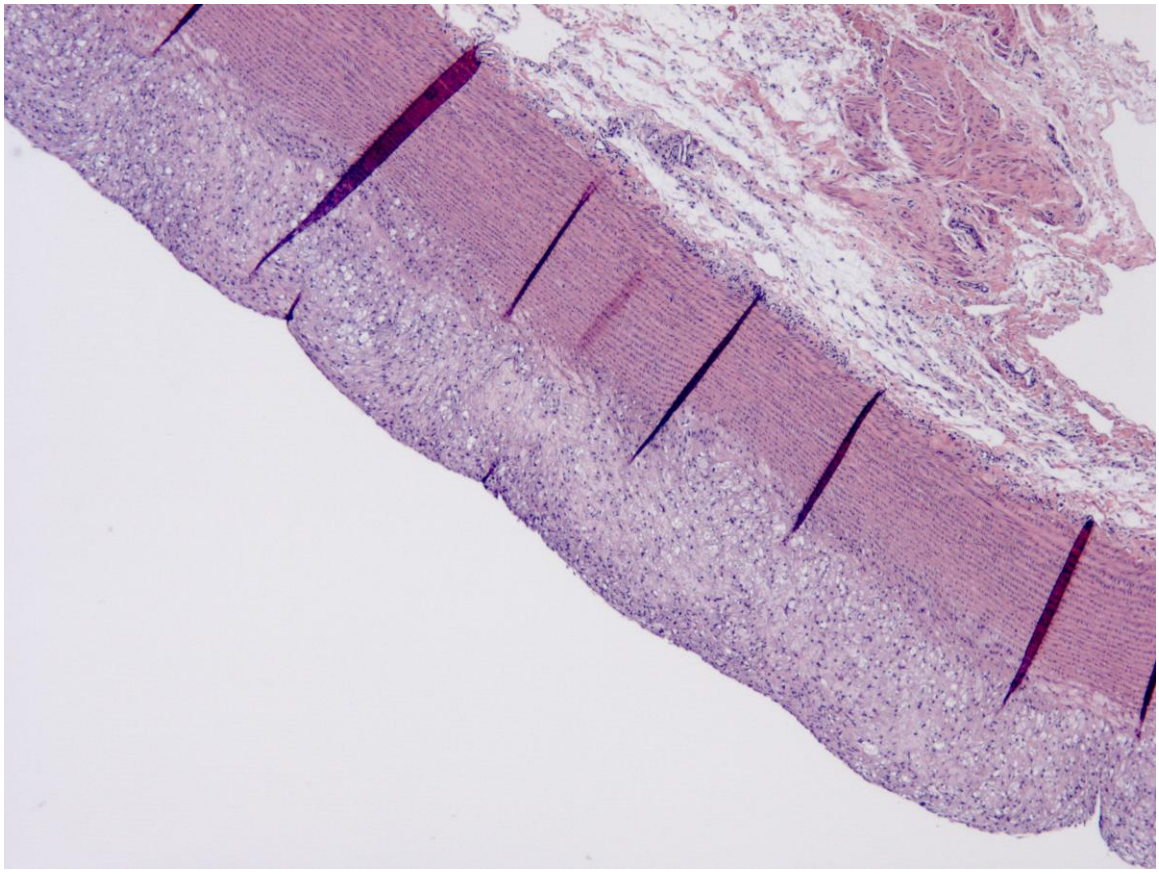
Picture 5B. *H&E Stain of Aorta* (*L449 Low Mg)*

Picture 6A. *Aortic Arch and Abdominal Aorta (L465 Low Mg)*

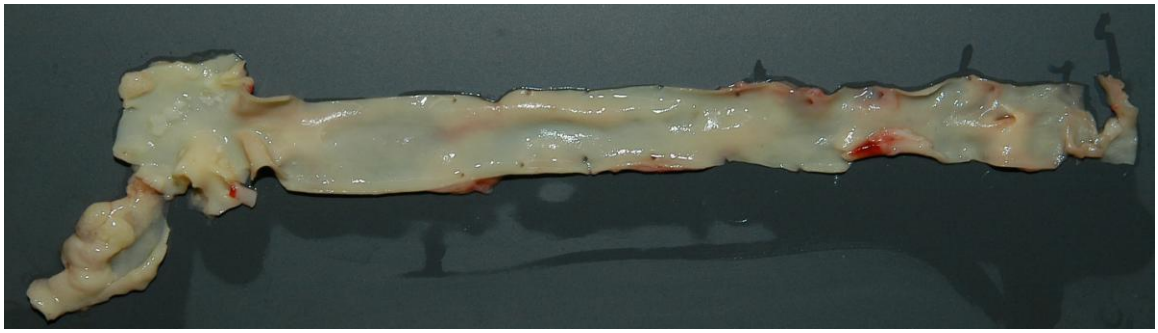


Picture 6B. *H&E Stain of Aorta* (*L465 Low Mg)*

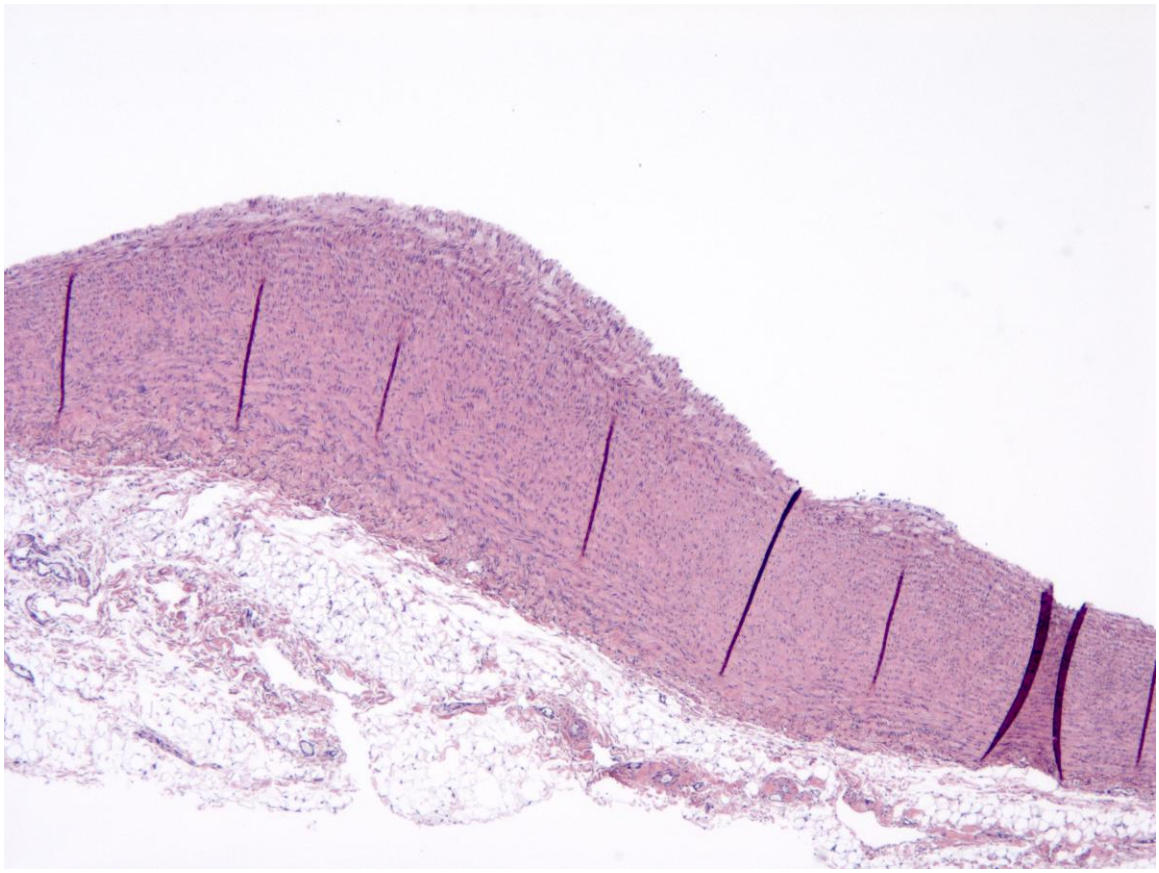
Picture 7A. *Aortic Arch and Abdominal Aorta (L470 Low Mg)*



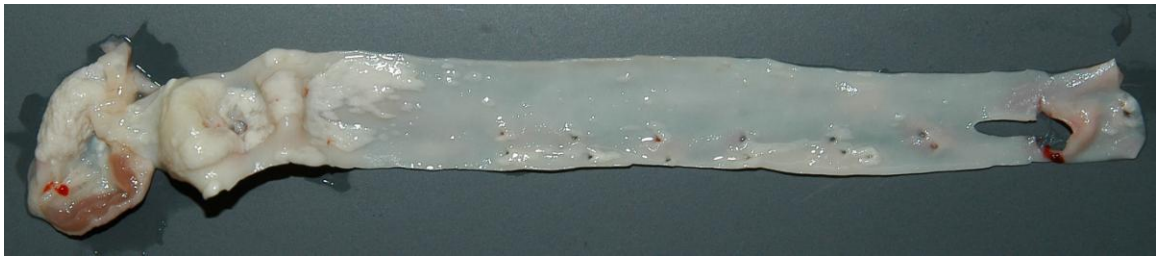
Picture 7B. *H&E Stain of Aorta* (*L470 Low Mg)*



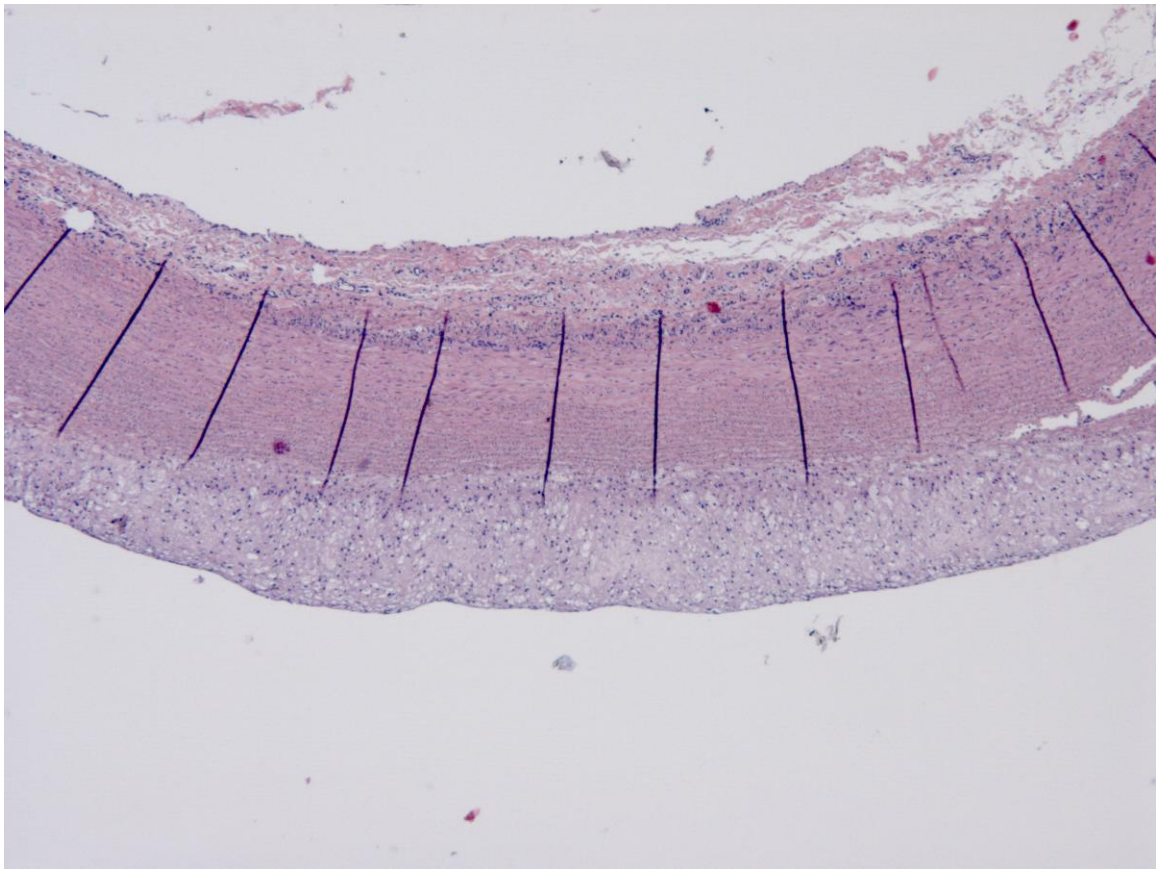
Picture 8A. *Aortic Arch and Abdominal Aorta (L452 Normal Mg)*



Picture 8B. *H&E Stain of Aorta* (*L452 Normal Mg)*

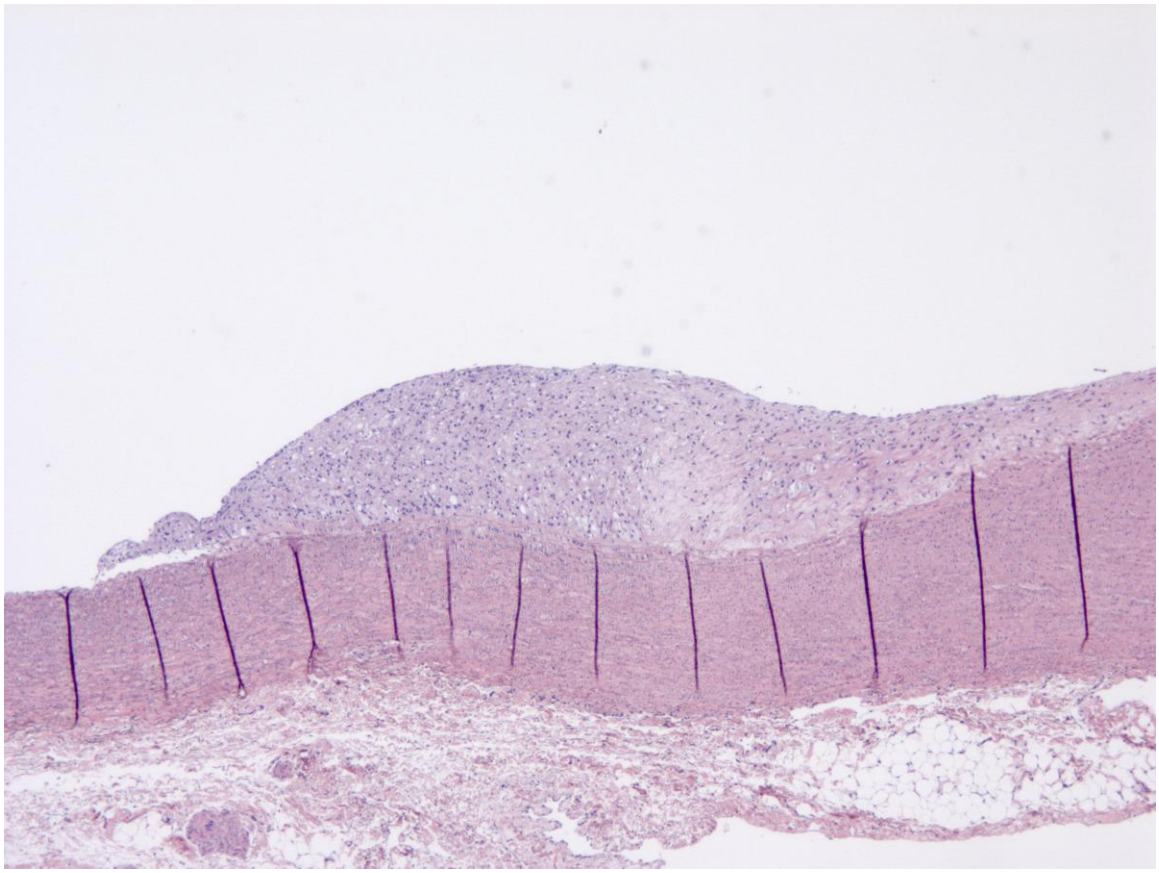


Picture 9A. *Aortic Arch and Abdominal Aorta (L457 Normal Mg)*

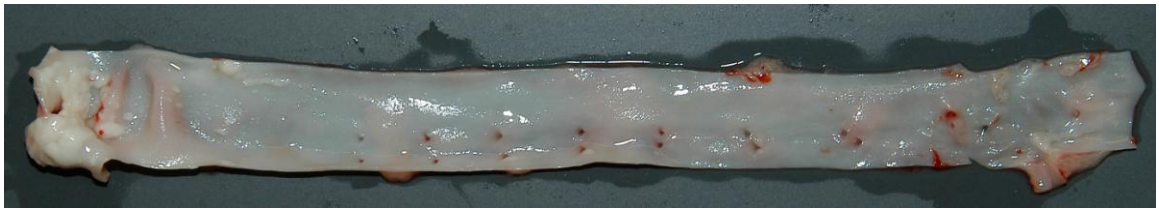


Picture 9B. *H&E Stain of Aorta* (*L457 Normal Mg)*

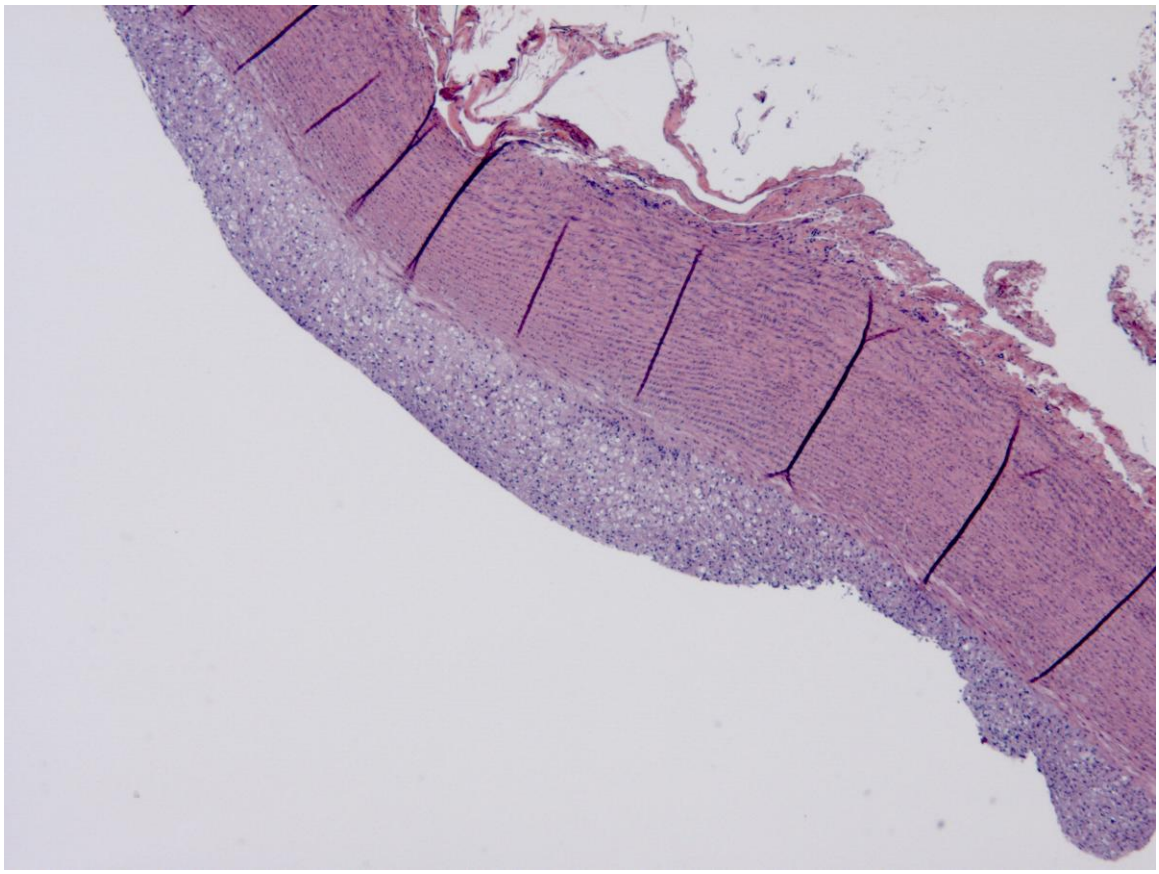
Picture 10A. *Aortic Arch and Abdominal Aorta (L458 Normal Mg)*



Picture 10B. *H&E Stain of Aorta* (*L458 Normal Mg)*

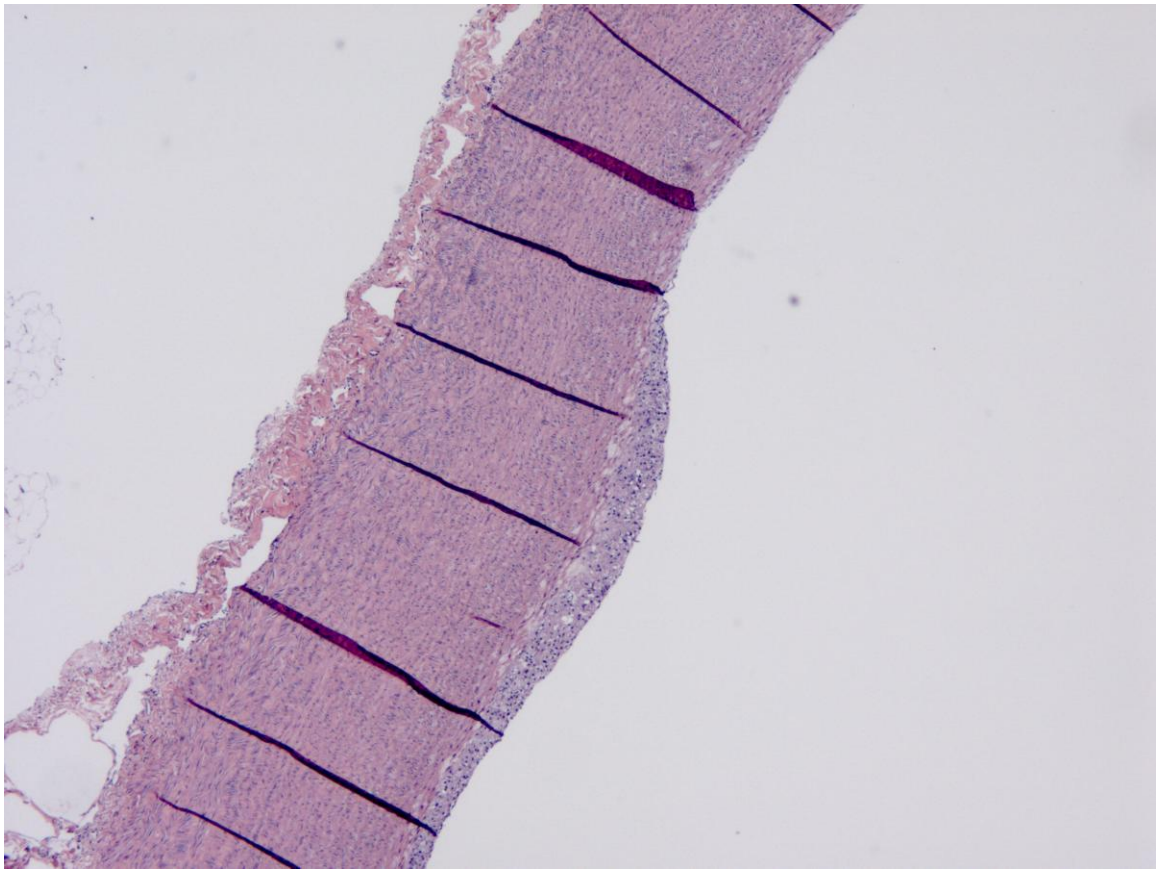


Picture 11A. *Aortic Arch and Abdominal Aorta (L461 Normal Mg)*



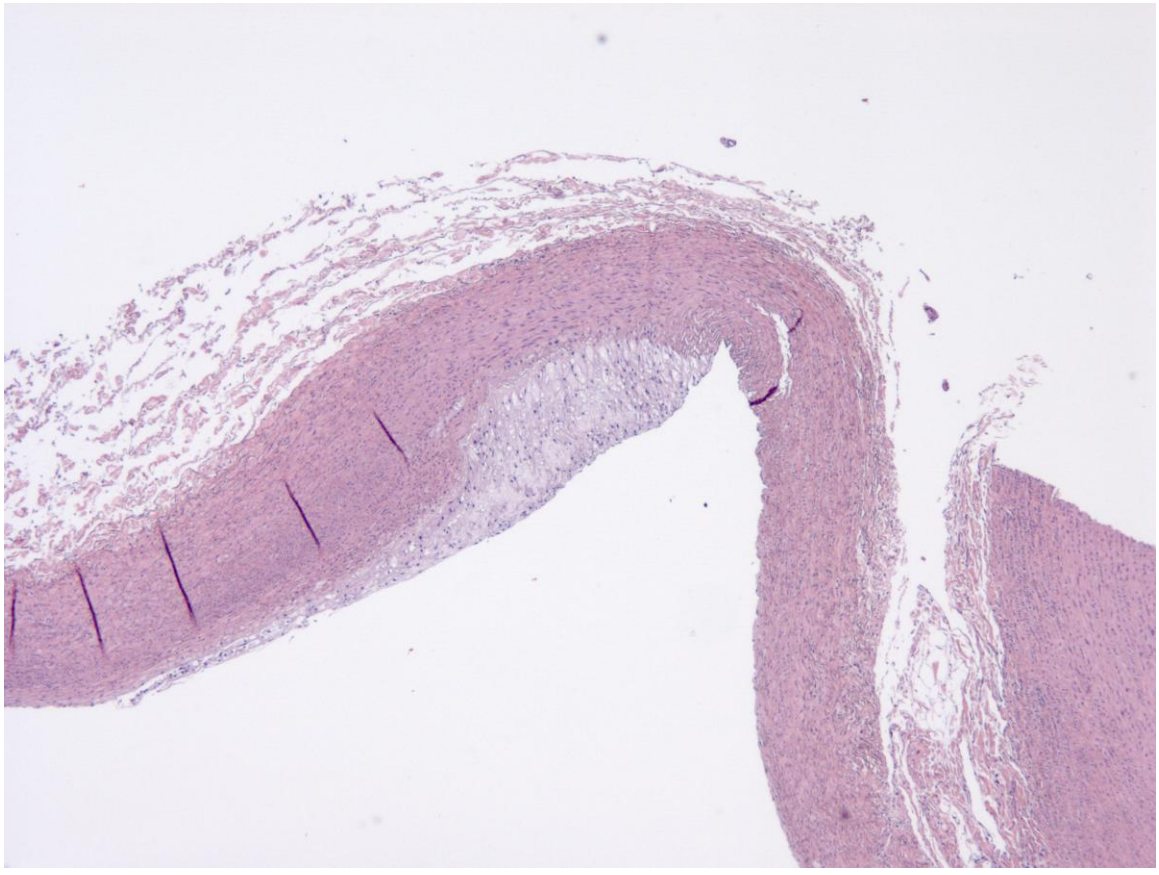
Picture 11B. *H&E Stain of Aorta* (*L461 Normal Mg)*

Picture 12A. *Aortic Arch and Abdominal Aorta (L466 Normal Mg)*



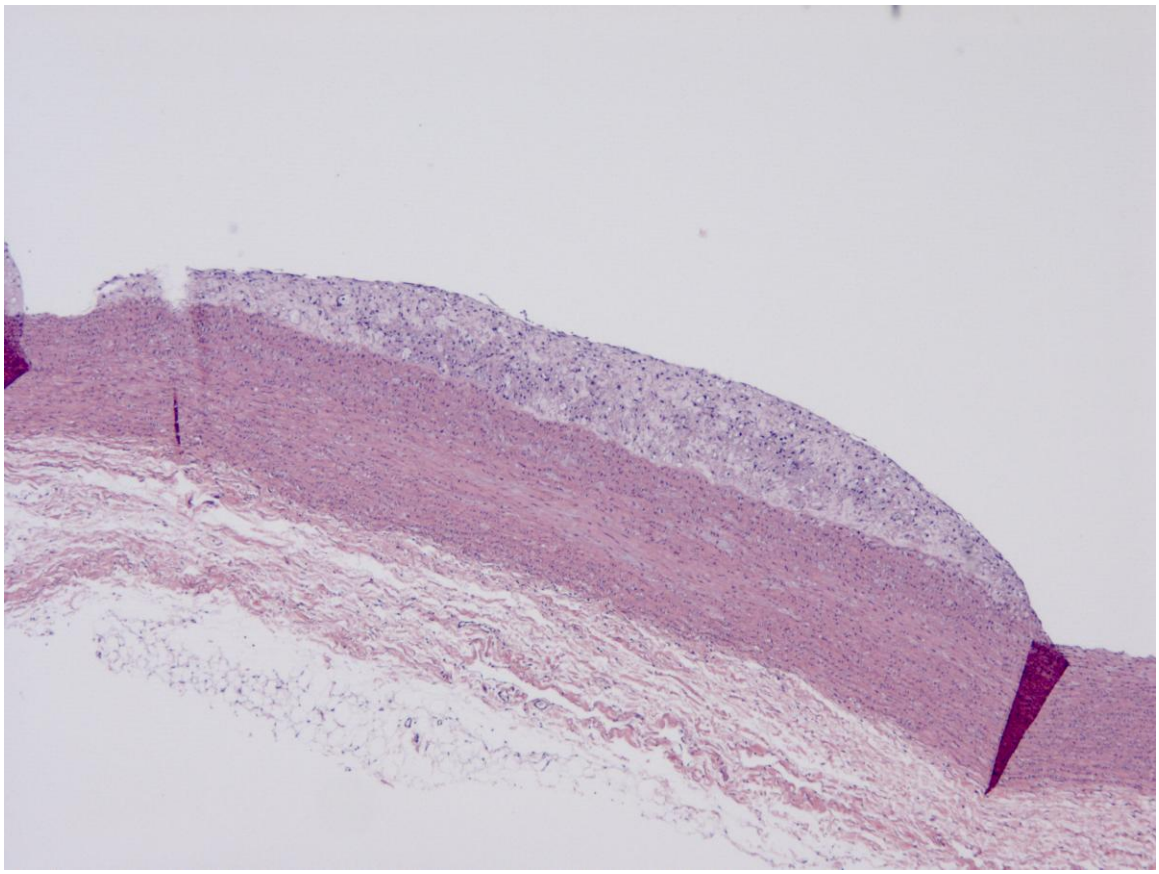
Picture 12B. *H&E Stain of Aorta* (*L466 Normal Mg)*

Picute 13A. *Aortic Arch and Abdominal Aorta (L471 Normal Mg)*

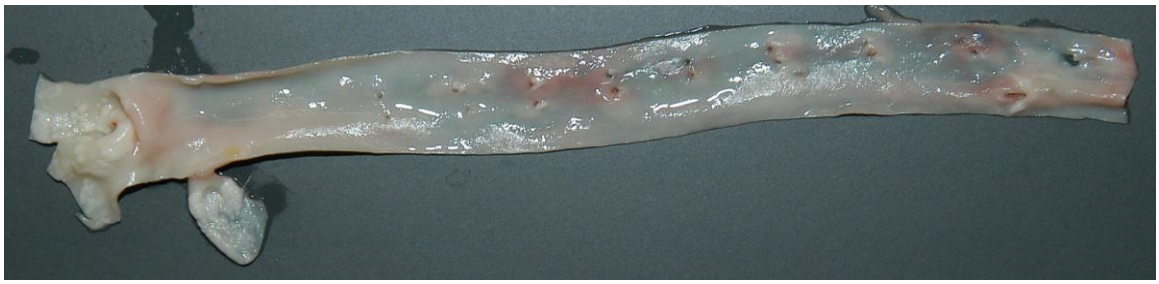


Picute 13B. *H&E Stain of Aorta* (*L471 Normal Mg)*

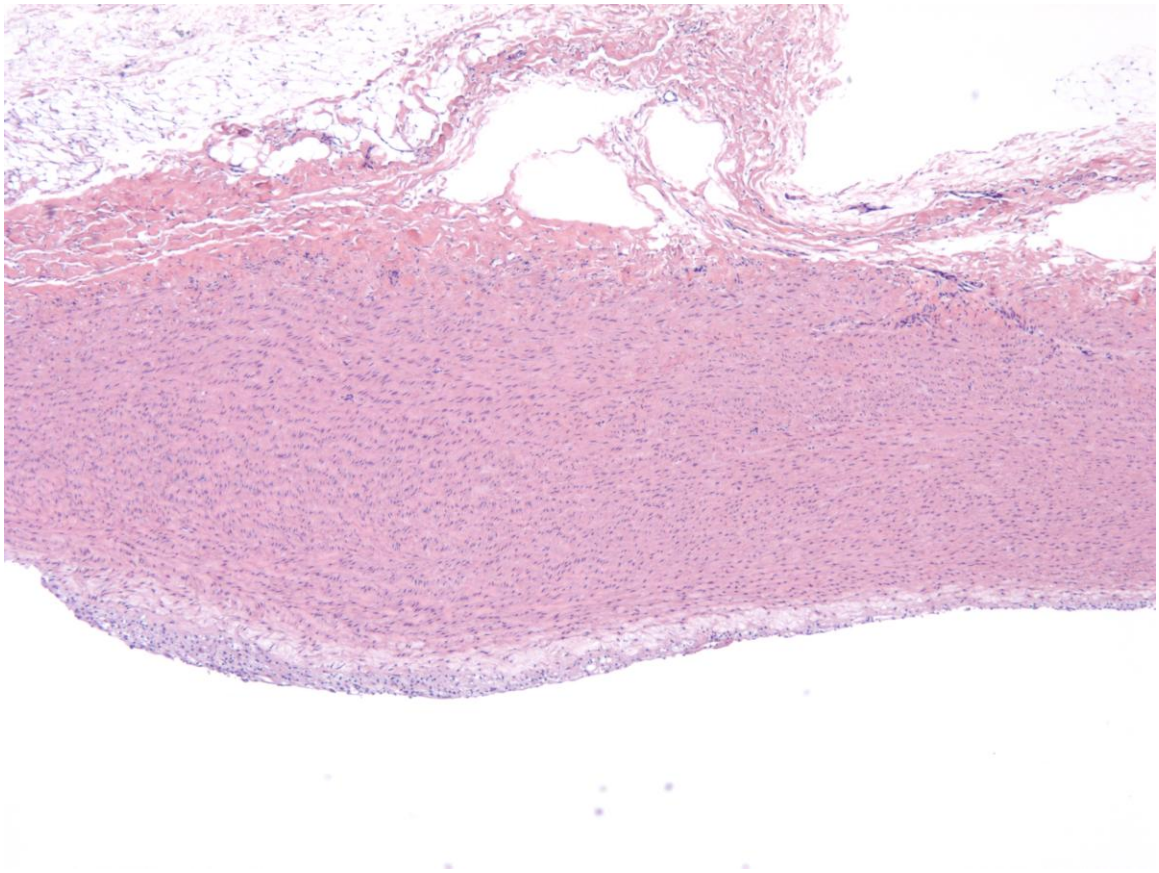
Picture 14A. *Aortic Arch and Abdominal Aorta (L472 Normal Mg)*



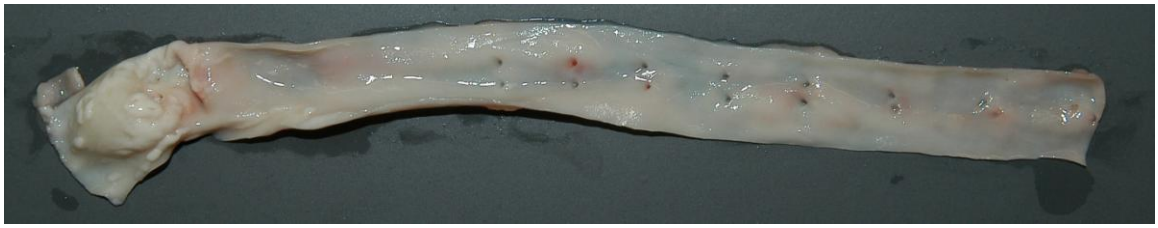
Picture 14B. *H&E Stain of Aorta* (*L472 Normal Mg)*



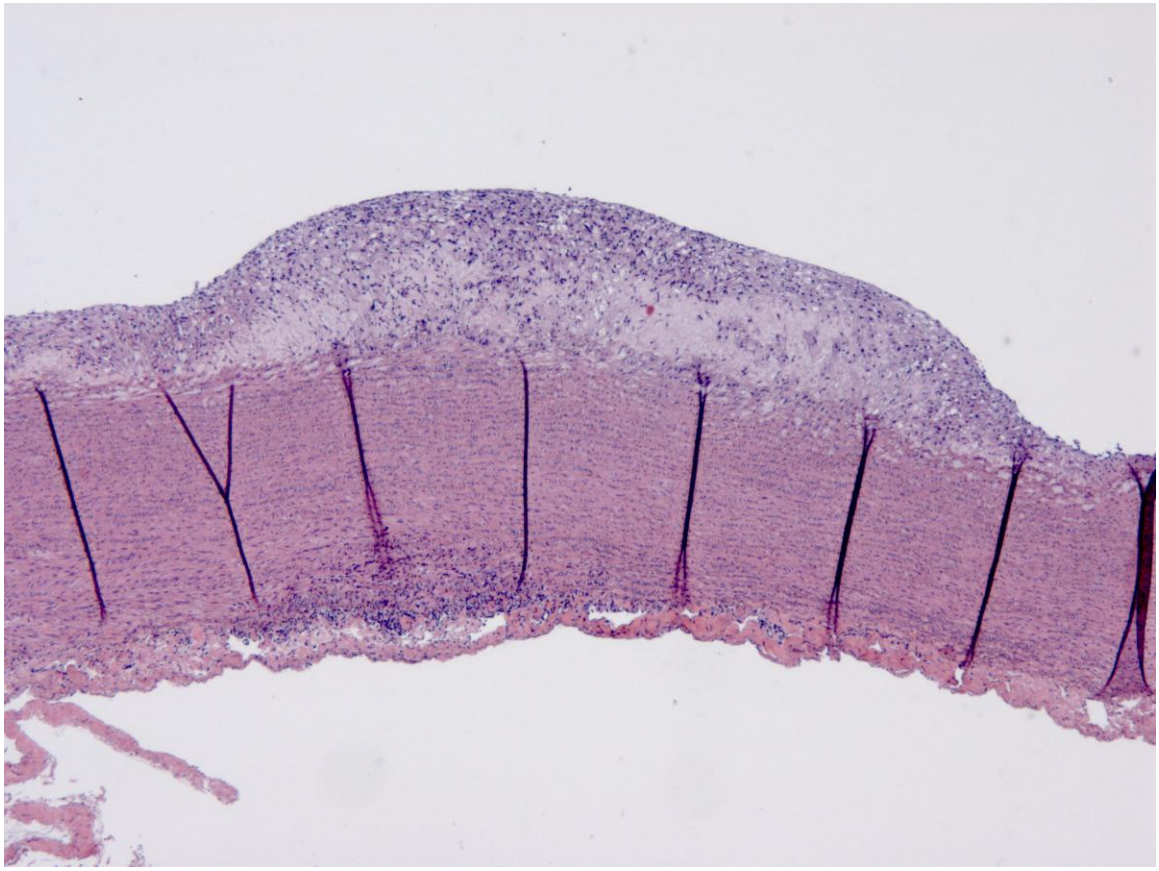
Picutre 15A. *Aortic Arch and Abdominal Aorta (L473 Normal Mg)*



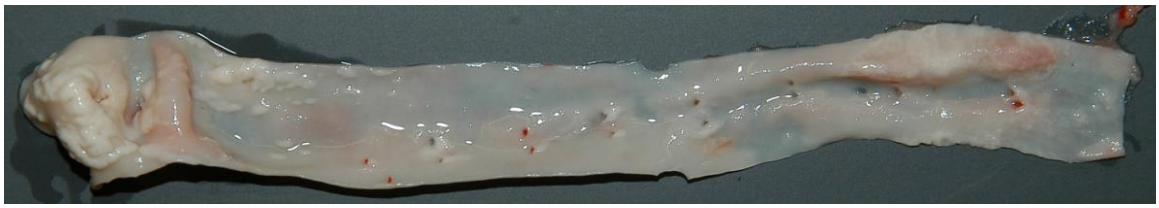
Picutre 15B. *H&E Stain of Aorta* (*L473 Normal Mg)*



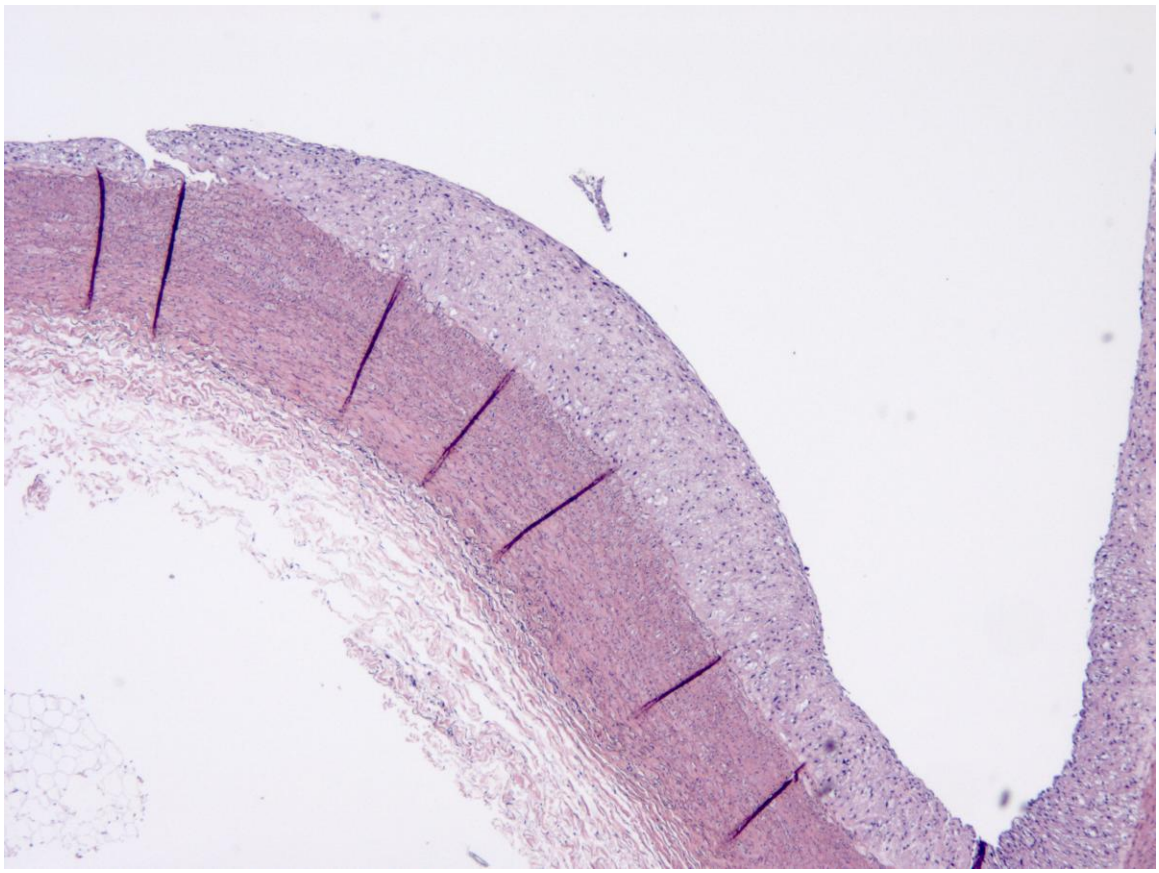
Picture 16A. *Aortic Arch and Abdominal Aorta (L455 High)*



Picture 16B. *H&E Stain of Aorta* (*L455 High)*

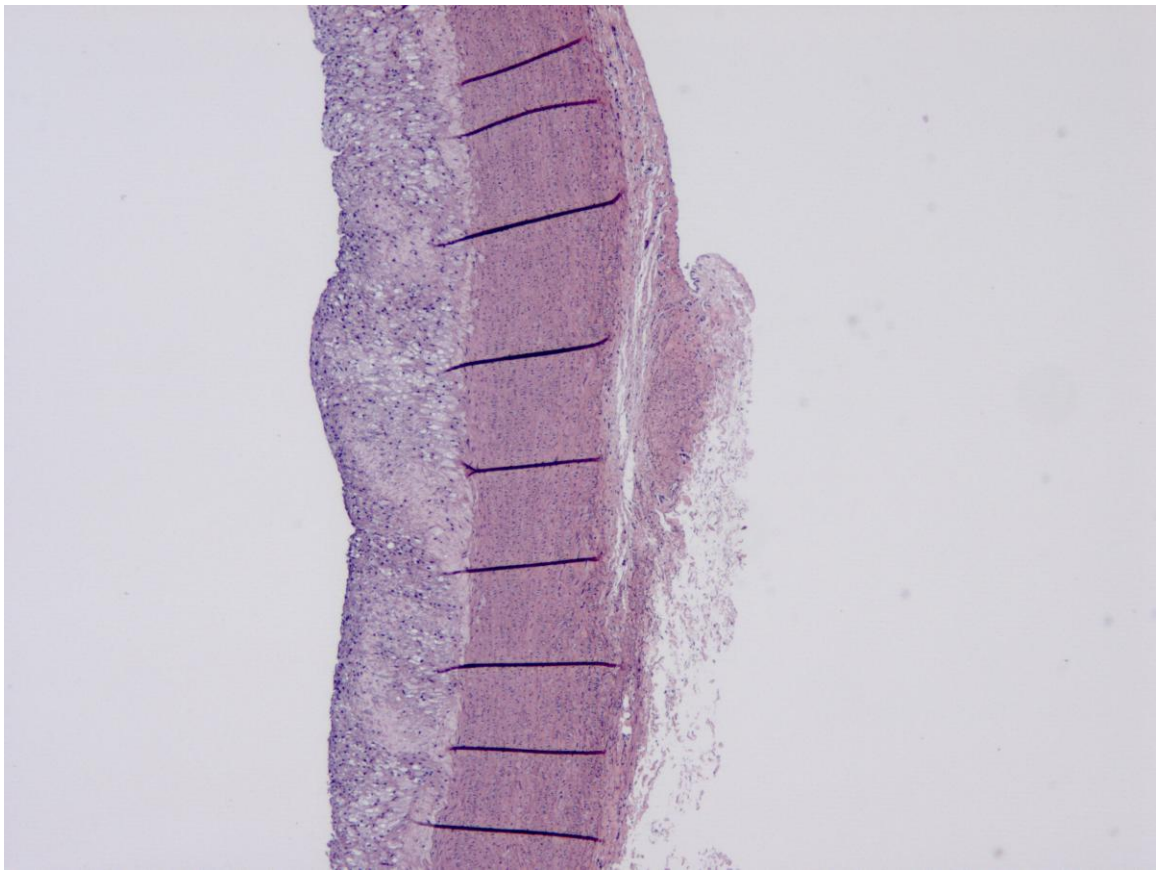


Picture 17A. *Aortic Arch and Abdominal Aorta (L459 High)*



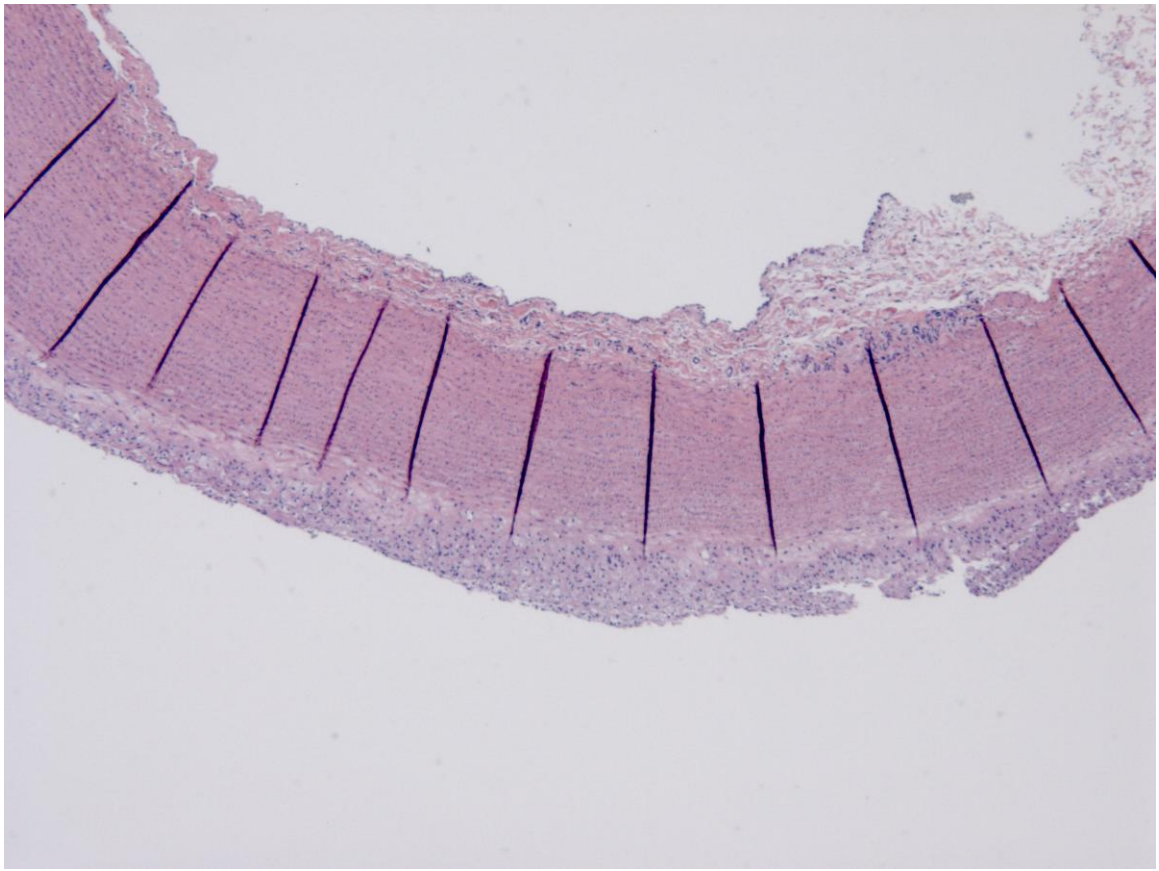
Picture 17B. *H&E Stain of Aorta* (*L459 High)*

Picture 18A. *Aortic Arch and Abdominal Aorta (L460 High)*



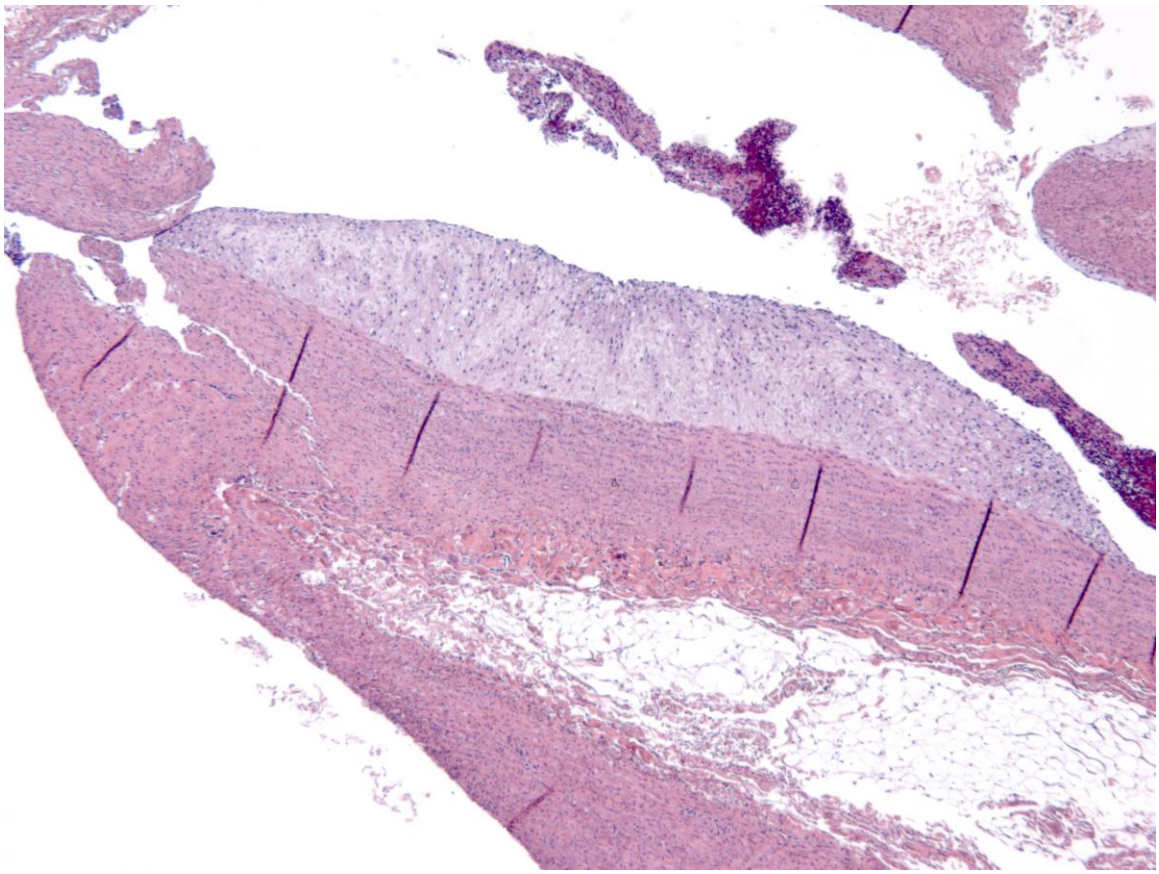
Picture 18B. *H&E Stain of Aorta* (*L460 High)*

Picture 19A. *Aortic Arch and Abdominal Aorta (L463 High)*

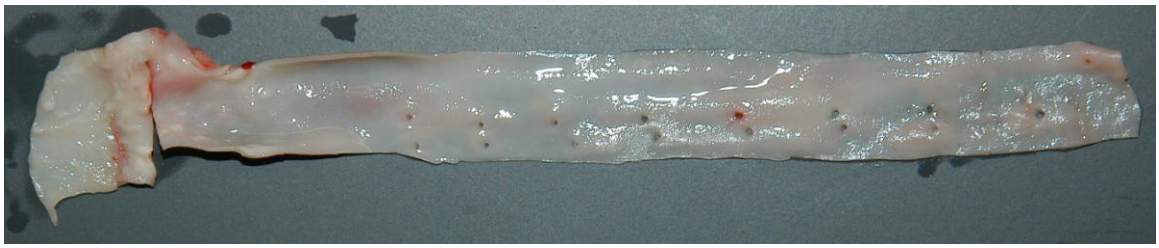


Picture 19B. *H&E Stain of Aorta* (*L463 High)*

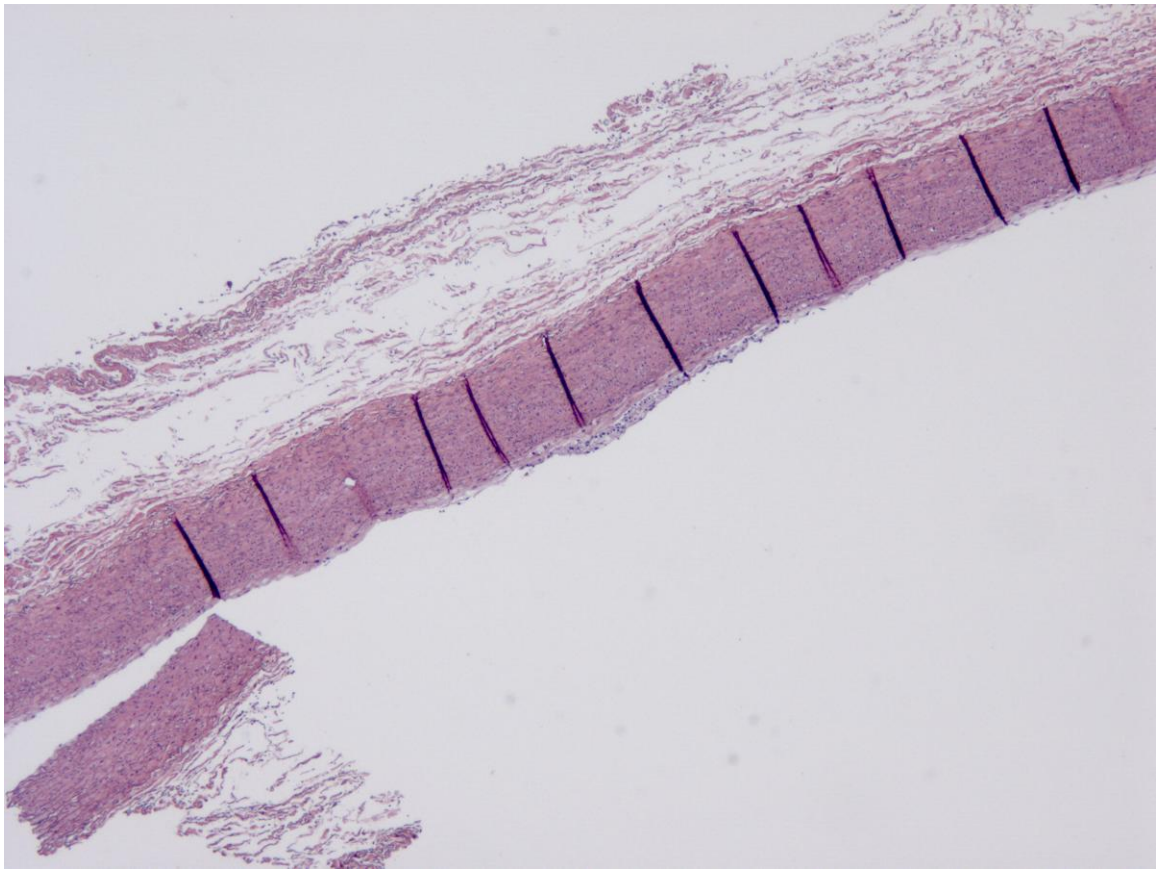
Picture 20A. *Aortic Arch and Abdominal Aorta (L464 High)*



Picture 20B. *H&E Stain of Aorta* (*L464 High)*

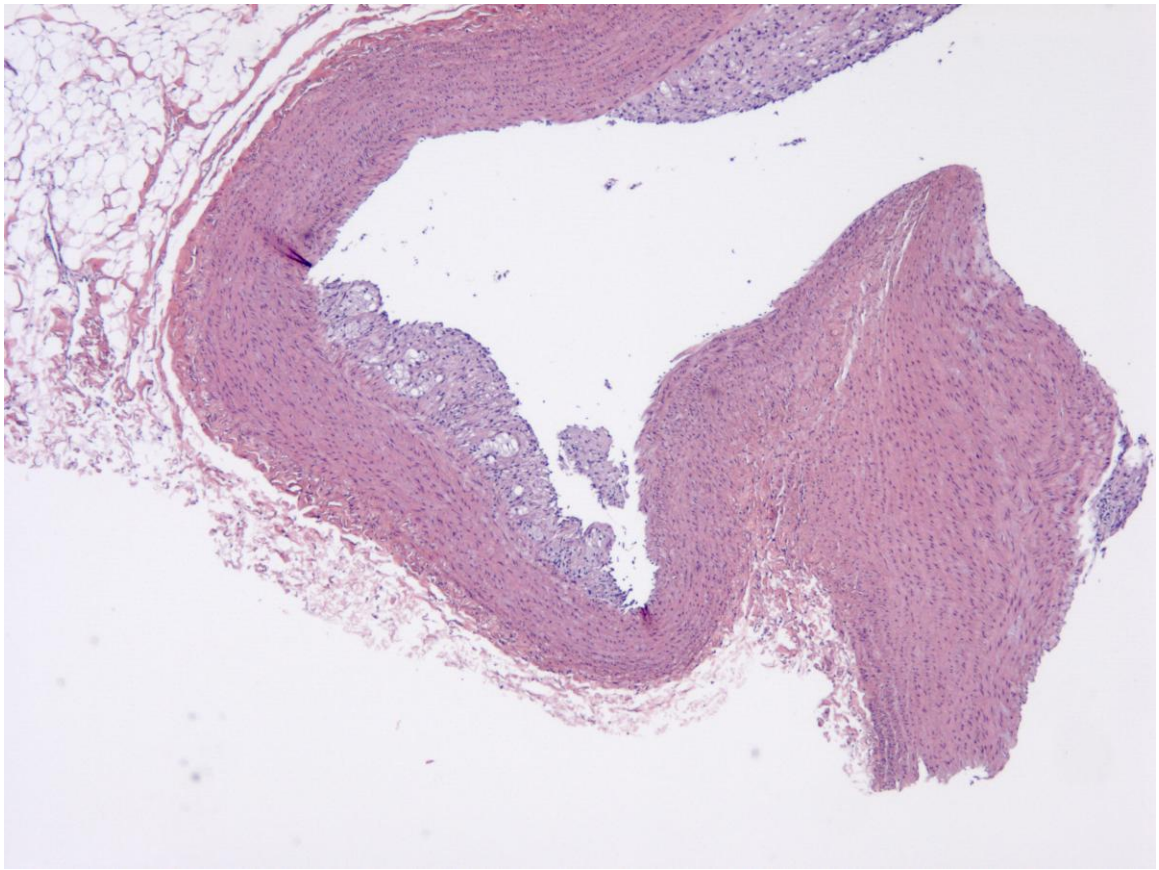


Picture 21A. *Aortic Arch and Abdominal Aorta (L467 High)*



Picture 21B. *H&E Stain of Aorta* (*L467 High)*

Picture 22A. *Aortic Arch and Abdominal Aorta (L469 High)*



Picture 22B. *H&E Stain of Aorta* (*L469 High)*

Serum Analysis

Total serum cholesterol did not differ by dietary treatment, although baseline values were slightly higher in the high Mg diet group (Table 10). Total serum cholesterol significantly increased over time in all treatment groups and reached approximately 1600 to 1800 mg/dL by 8 weeks (Figure 5).

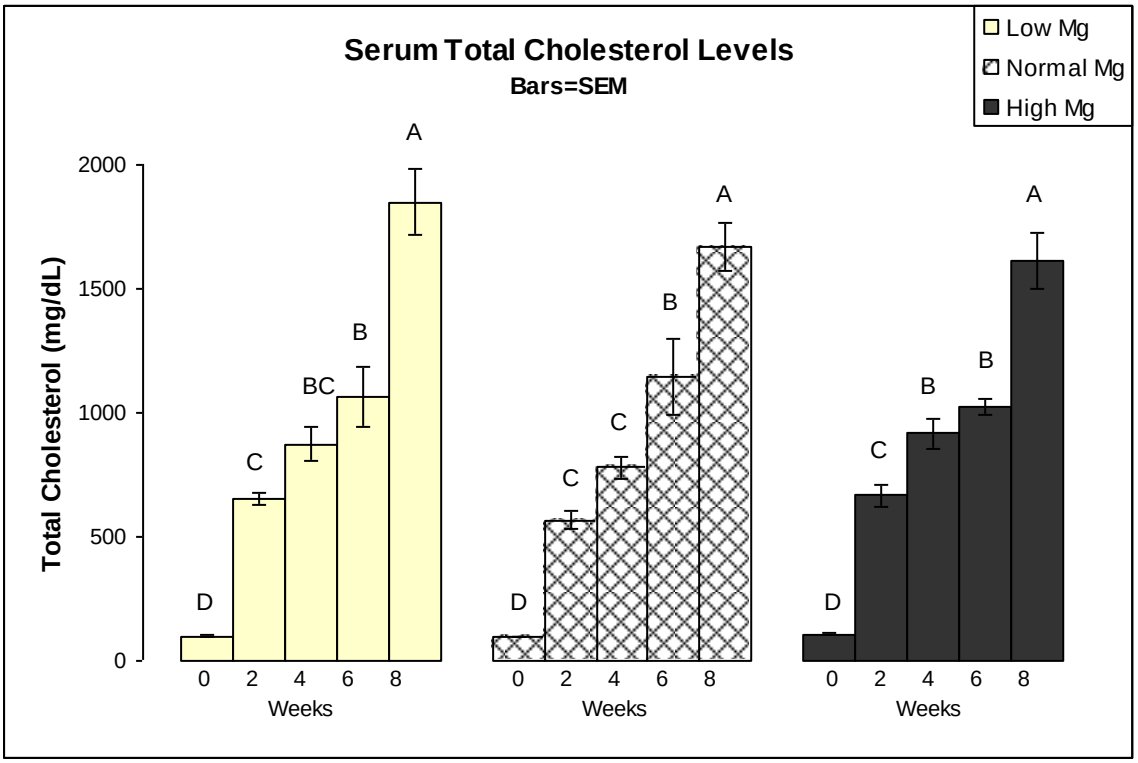


Figure 5. *Serum Total Cholesterol Levels (mg/dL)*
Mean ± SEM
Letters indicate difference between timepoints within dietary treatment ($p \leq 0.05$)

LDL values differed significantly between treatment groups at 2 weeks only. The High Mg group had the highest level of LDL, the Normal Mg group the lowest, and the Low Mg group did not differ from either the High or Normal group (Table 10). There was a steady increase in LDL values for all groups, reaching approximately 1400 to 1600 mg/dL by 8 weeks (Figure 6).

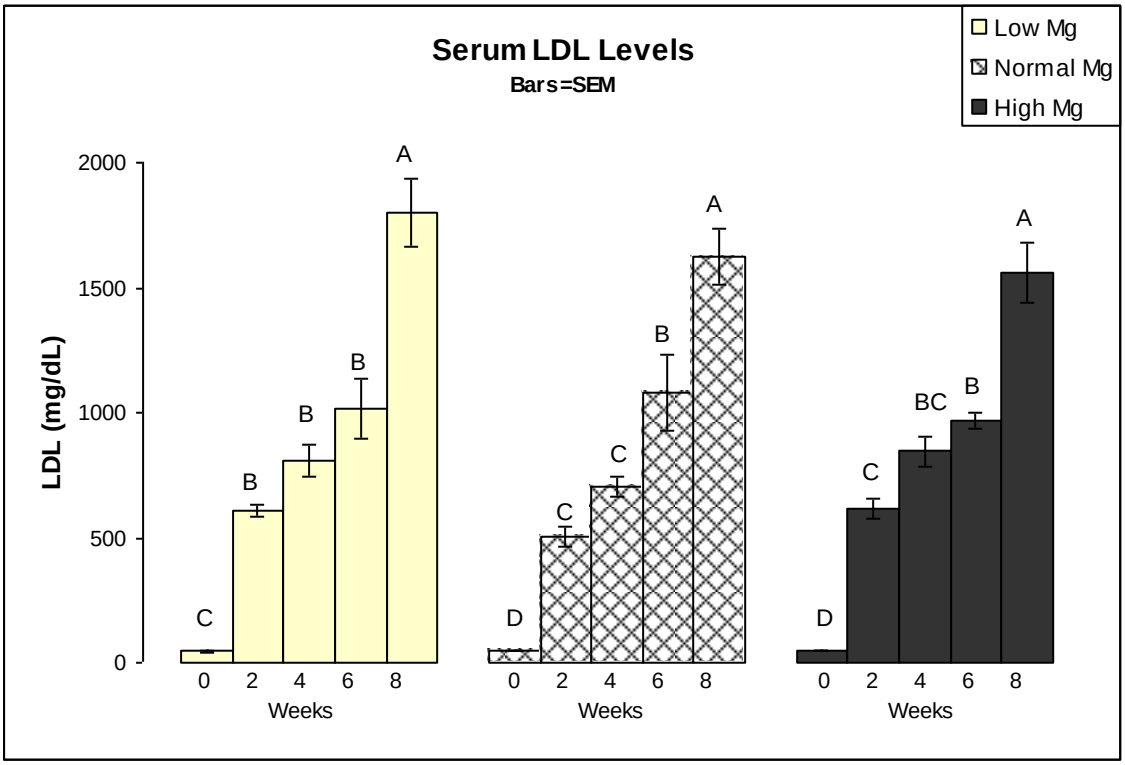


Figure 6. *Serum LDL Cholesterol Levels (mg/dL)*
Mean ± SEM
Letters indicate difference between timepoints within dietary treatment ($p \leq 0.05$)

HDL levels did not change significantly over time (Table 10). Small flucuations caused a significant difference between the low Mg and normal Mg groups at 2 and 6 weeks (Figure 7) but neither group ever differed significantly from the high Mg group.

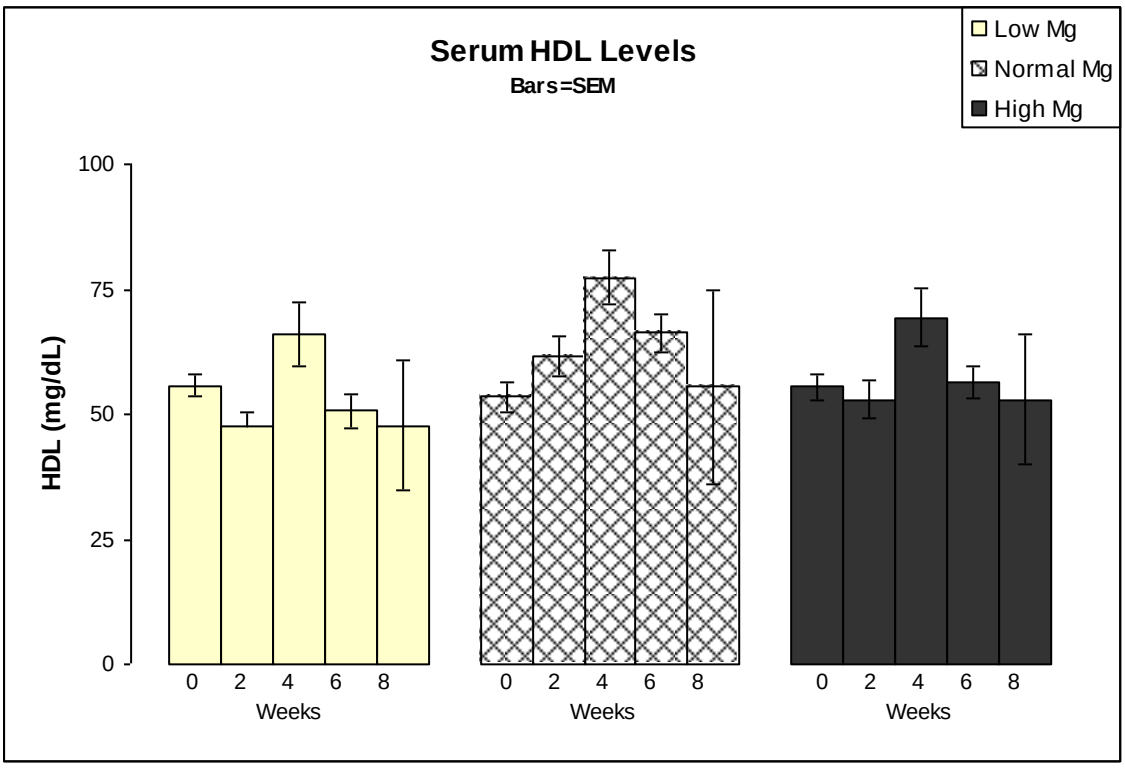


Figure 7. *Serum HDL Cholesterol Levels (mg/dL) Mean ± SE*

For all three treatment groups, the LDL:HDL ratio did not significantly differ from baseline until the 8 week timepoint (Figure 8). At the 2 and 4 week timepoints the treatment groups differed from each other, with the normal Mg group having a significantly lower LDL:HDL ratio then the low Mg and the high Mg groups (Table 10).

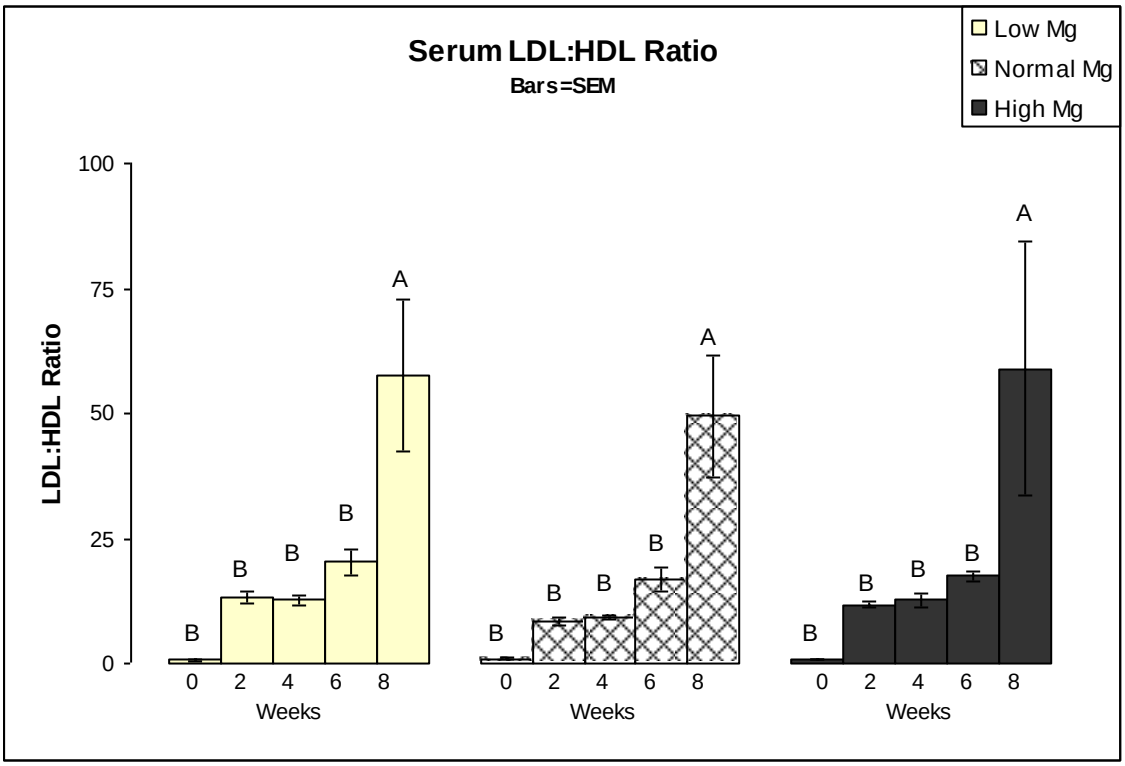


Figure 8. *Serum LDL:HDL Ratio*
Mean ± SEM
Letters indicate difference between timepoints within dietary treatment ($p \leq 0.05$)

Serum TG levels did not change signicantly in the normal Mg group (Figure 9). In the high and low Mg groups, TG levels rose over time, reaching a maximum at 6 weeks in both groups and then decreasing at 8 weeks (Table 10). Only at the 6 week timepoint was there an effect of dietary treatment with the low Mg and normal Mg groups differing significantly. The high Mg group did not significantly differ from either the low Mg or the normal Mg group at any timepoint.

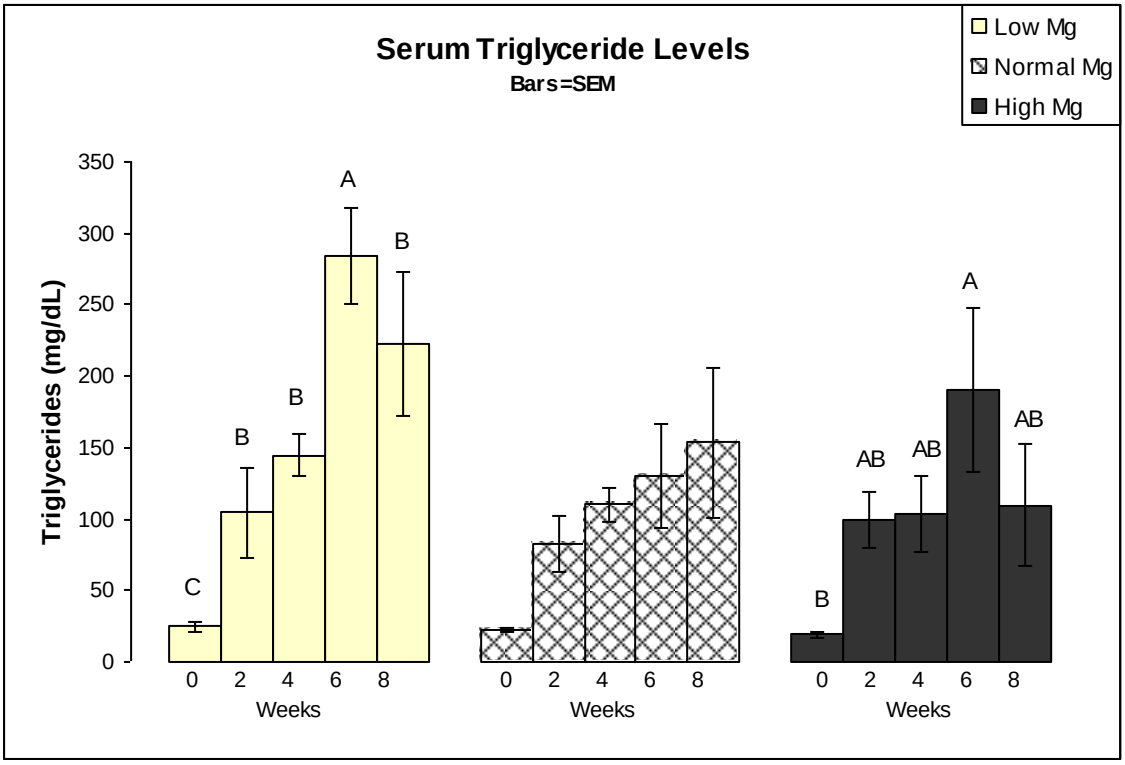


Figure 9. *Serum Triglyceride Levels (mg/dL)*
Mean ± SEM
Letters indicate difference between timepoints within dietary treatment ($p \leq 0.05$)

		Baseline	2 weeks	4 weeks	6 weeks	8 weeks
Total	Low	100 ± 2[b]	653 ± 25*	875 ± 68*	1066 ± 120*	1849 ± 133*
	Normal	98 ±1[b]	566 ± 37*	780 ± 42*	1144 ± 153*	1673 ± 97*
	High	111± 8[a]	668 ± 44*	916 ± 62*	1024 ± 32*	1612 ± 112*
LDL	Low	47 ± 3	605 ± 24* [ab]	809 ± 65*	1015 ± 119*	1801 ± 137*
	Normal	47 ± 2	505 ± 37* [b]	702 ± 38*	1078 ± 156*	1624 ± 109*
	High	48 ± 3	616 ± 42* [a]	847 ± 60*	967 ± 31*	1559 ± 120*
HDL	Low	56 ± 2	48 ± 3 [b]	66 ± 6	51 ± 4 [b]	48 ± 13
	Normal	53 ± 3	62 ± 4 [a]	77 ± 5	66 ± 4 [a]	56 ± 19
	High	55 ± 3	53 ± 4 [ab]	69 ± 6	56 ± 3 [ab]	53 ± 13
LDL:HDL	Low	0.7 ± 0.2	13 ± 1 [a]	13 ± 1 [a]	20 ± 3	58 ± 15*
	Normal	0.9 ± 0.1	8 ± 1 [b]	9 ± 0 [b]	17 ± 2	50 ± 12*
	High	0.9 ± 0.1	12 ± 1 [a]	113 ± 1 [a]	17 ± 1	59 ± 25*
TG	Low	25 ± 4	105 ± 31*	144 ± 14*	284 ± 34* [a]	222 ± 51*
	Normal	22 ± 2	83 ± 20	110 ± 12	130 ± 36 [b]	153 ± 52
	High	19 ± 2	99 ± 19*	104 ± 26	190 ± 57* [ab]	110 ± 43

Table 10. *Serum Lipid Values (mg/dL)*
Mean ± SEM
**=significantly different from baseline ($p \leq 0.05$)*
Letters within a column indicate difference between dietary treatment at same timepoint ($p \leq 0.05$)

Serum Mg levels in the low Mg group decreased significantly at 2 weeks and then increased until levels were significantly higher than baseline at 8 weeks ($p \leq 0.05$) (Figure 10 and Table 11). Serum Mg steadily increased in the normal Mg and high Mg groups and were statistically higher than baseline levels at 4, 6, and 8 weeks.

At 2 weeks, the high Mg group had higher serum Mg than both the normal and low Mg groups (Figure 11). At 4 and 6 weeks the high Mg group had the highest serum Mg and the low Mg group had the lowest, with the normal Mg group not differing from either one. By 8 weeks serum Mg levels stabilized and were not significantly different between any dietary treatment.

Diet	Week				
	0	2	4	6	8
Low	2.9 ± 0.2	2.6 ± 0.1* [b]	3.0 ± 0.1 [b]	3.6 ± 0.2 [b]	3.8 ± 0.3*
Normal	2.8 ± 0.1	2.9 ± 0.1 [b]	3.4 ± 0.2* [ab]	3.7 ± 0.2* [ab]	4.1 ± 0.3*
High	2.8 ± 0.1	3.4 ± 0.2 [a]	4.1 ± 0.4* [a]	4.3 ± 0.2* [a]	4.3 ± 0.3*

Table 11. *Serum Mg Levels (mg/dL)*
Mean ± SEM
** = significantly different from baseline ($p \leq 0.05$)*
Letters indicate difference between dietary treatment at same timepoint ($p \leq 0.05$)

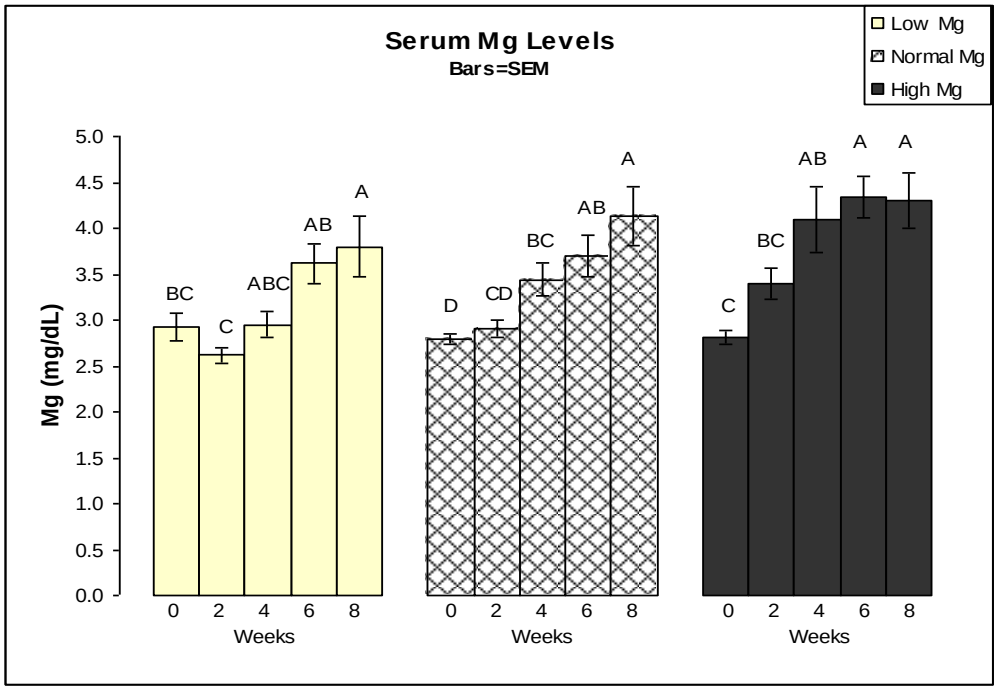


Figure 10. *Serum Mg Levels (mg/dL)*
Mean ± SEM
Letters indicate difference between timepoints within dietary treatment (p≤0.05)

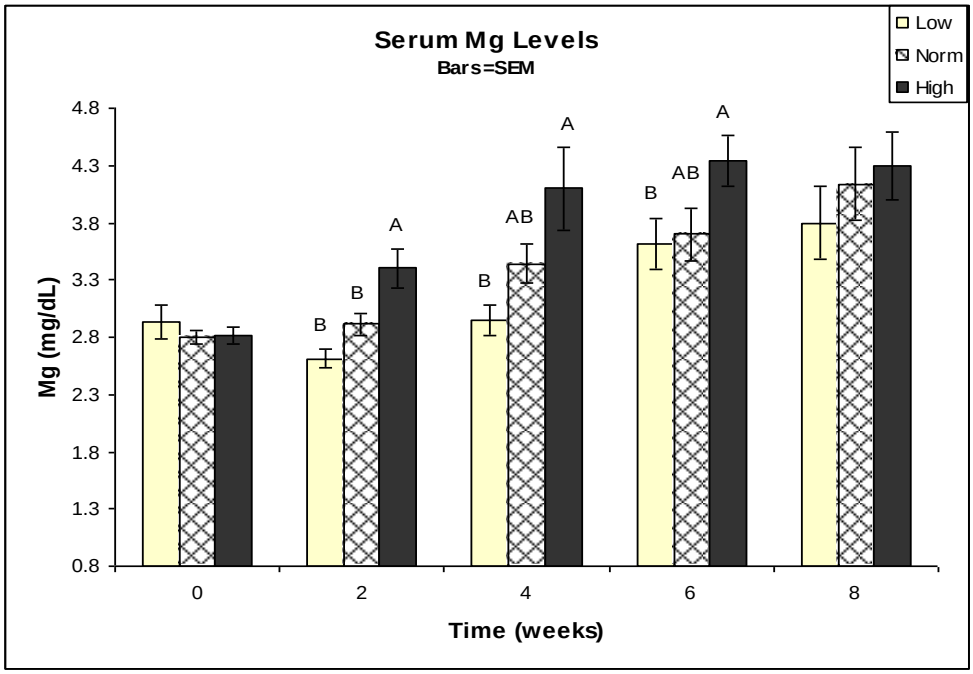


Figure 11. *Serum Mg Levels (mg/dL)*
Mean ± SEM
Letters indicate difference between dietary treatment at same timepoint (p≤0.05)

In all treatment groups, CRP increased significantly at 4 and 6 weeks but returned to baseline levels by 8 weeks (Table 12 and Figure 12). CRP levels did not differ between diet groups at any timepoint.

Diet	Week				
	0	2	4	6	8
Low	1.06 ± 0.05	1.03 ± 0.09	1.28 ± 0.04*	1.24 ± 0.06*	1.12 ± 0.04
Normal	1.01 ± 0.08	1.14 ± 0.06	1.28 ± 0.02*	1.32 ± 0.03*	1.11 ± 0.02
High	1.02 ± 0.12	1.04 ± 0.12	1.26 ± 0.02*	1.26 ± 0.04*	1.10 ± 0.02

Table 12. *Serum CRP levels (mg/L)*
Mean ± SEM
** = significantly different from baseline (p≤0.05)*

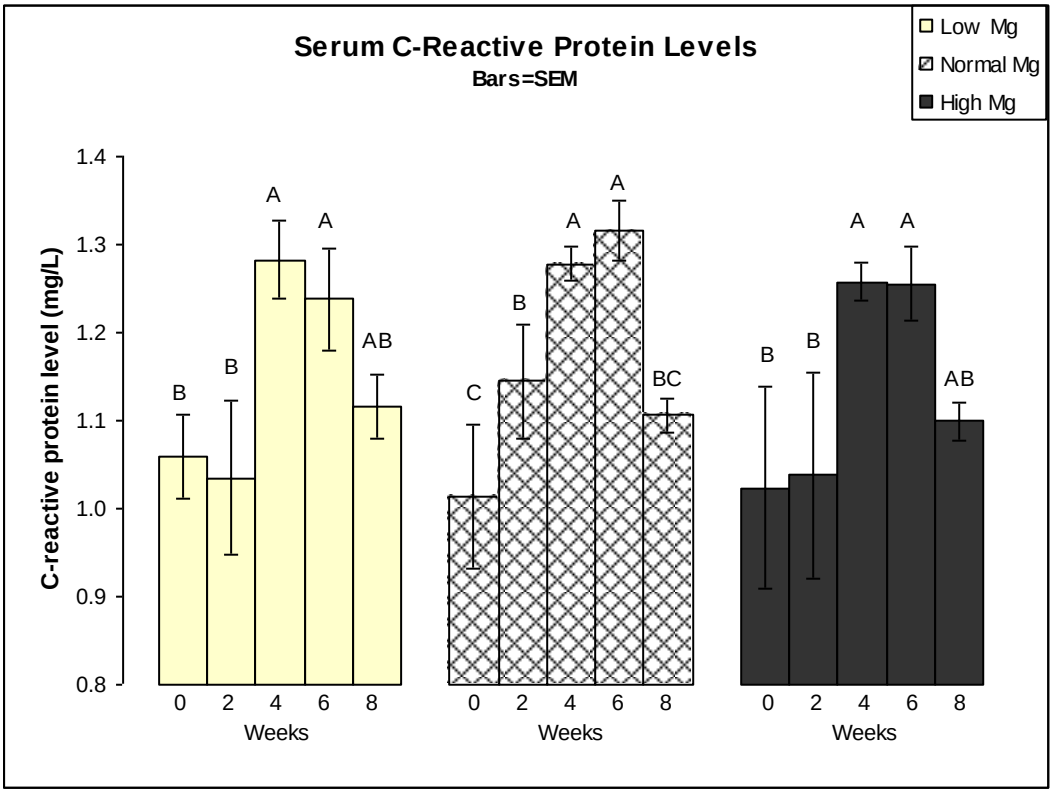


Figure 12. *Serum CRP levels (mg/L)*
Mean ± SEM
Letters indicate difference between timepoints within dietary treatment (p≤0.05)

Serum s-TM levels did not signicantly differ at any time point and did not differ between treatment groups.

Diet	Week				
	0	2	4	6	8
Low	12.4 ± 1.1	12.6 ± 1.4	12.8 ± 0.8	11.0 ± 0.2	11.8 ± 0.7
Normal	11.9 ± 1.2	13.4 ± 2.8	12.2 ± 1.4	11.6 ± 0.6	11.2 ± 0.5
High	12.0 ± 0.9	13.8 ± 2.5	13.2 ± 1.8	11.4 ± 0.3	11.6 ± 0.4

Table 13. *Serum s-Thrombomodulin levels (ng/mL)*
Mean ± SEM

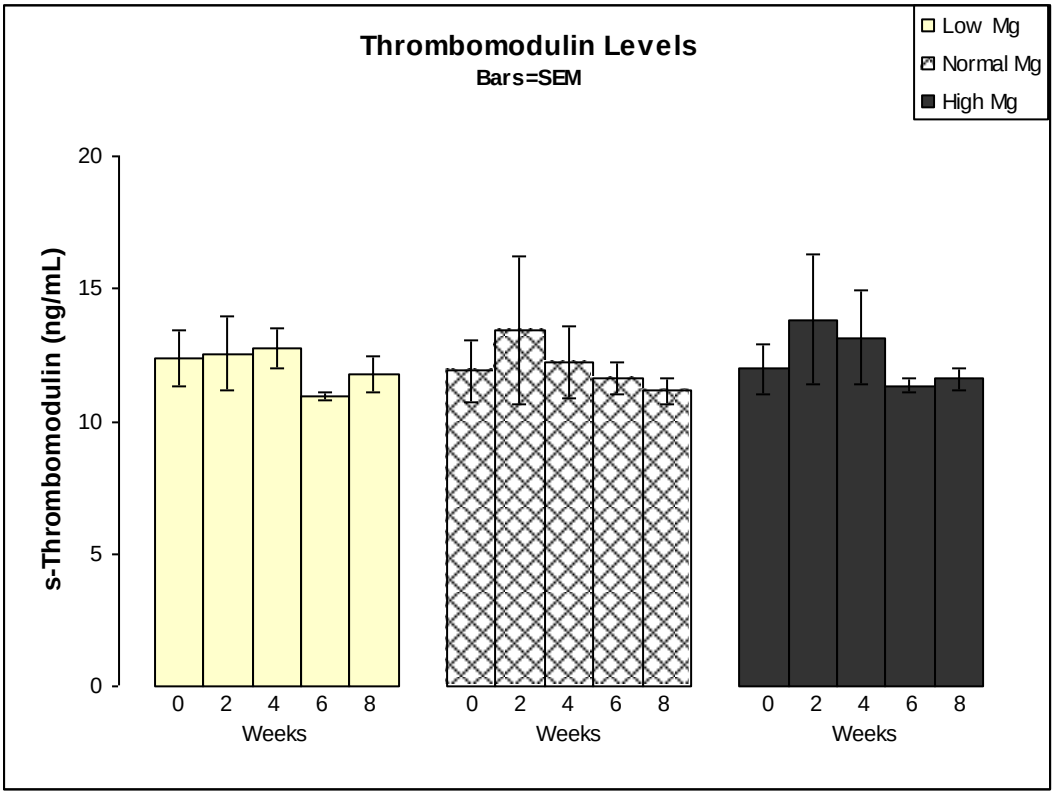


Figure 13. *Serum TM (ng/mL)*
Mean ± SEM

CHAPTER 4: DISCUSION/CONCLUSIONS

The goal of the study was to determine if dietary levels of Mg would influence plaque development in rabbits. I had hypothesized that atherosclerotic plaque, serum cholesterol, a marker of inflammation (CRP), and a marker of endothelial damage (sTM) would all inversley correlate to Mg intake in a dose-dependent manner (Low Mg > Normal Mg > High Mg).

At the end of the study, the high Mg group had the lowest total cholesterol (1612 mg/dL), lowest LDL cholesterol (1559 mg/dL), and the lowest TG levels (110 mg/dL), although these were not statistically different from the other groups. The low Mg group had the highest total cholesterol (1849 mg/dL), highest LDL cholesterol (1801 mg/dL), and the highest TG levels (222 mg/dL), although they were not statistically different from the other groups. Although total cholesterol, LDL, HDL, and TG levels were not seen to significantly differ in this study, it is possible that the more atherogenic fractions of cholesterol were different among treatment groups. I did not measure the most atherogenic lipo-proteins, including oxidized-LDL and β-VLDL (37). It is possible that the low Mg group had a larger proportion of the LDL as modified, more atherogenic lipo-proteins.

The Low Mg group had the highest amount of plaque accumulation. This was found in gross examination of the aortas, and was confirmed by histological analysis of the aorta intimal thickness. The Normal and High Mg groups did not differ in either gross examination of plaque or in histological analysis of intimal thickness.

One of the most confounding results of this study is the fact that feed intake differed significantly among diet groups. It is interesting to note, however, that while the normal and high Mg groups had significant differences in feed intakes (average of 59 g/d vs 77 g/d respectively) their plaque development did not differ. The low Mg group while having feed intake (64 g/d) not statistically different from either the high or normal Mg groups had much more plaque accumulation.

Serum Mg levels increased in all groups, which is surprising considering that the Mg levels of the diet in the low Mg group (0.11%) were much lower than the standard maintenance chow (0.19%). The low Mg group had a significant decrease in serum Mg at 2 weeks (-0.3 mg/dL) and then serum levels rose and were eventually significantly greater than baseline levels at 8 weeks (+0.9 mg/dL). Serum Mg levels increased in the normal and high Mg groups and were significantly different from baseline at 4,6, and 8 weeks. The levels of serum Mg seen were slightly higher than normal parameters for rabbits of 2.2-4.0 mg/dL (47) at the end of the trial in the normal Mg group and 4, 6, and 8 weeks for the high Mg group. This increase in serum Mg in cholesterol fed rabbits has been previously reported and Altura et al. (45) proposed that this was due to influx of Mg into the serum from tissue pools. Since Mg is neccesary for cholesterol metabolism, it is reasonable to hypothesize that more Mg was needed in the serum to handle the large loads of cholesterol present. The fact that serum Mg in the Low Mg group was within normal ranges at all times, yet plaque development was increased, supports the findings in previous literature that serum Mg measurement alone is not adequete to determine risk for CVD.

Although in humans serum CRP levels have been seen to correlate with serum Mg (38), this study showed no correlation between dietary Mg intake or serum Mg with CRP. CRP increased from baseline levels in all groups at 4 and 6 weeks, was not significantly different from baseline by 8 weeks, and dietary treatment did not affect CRP levels. This transitory effect is probably due to the fact that CRP is an acute phase inflammtory marker (40). The increase CRP in all groups supports previous literature that found that feeding rabbits high levels of fat and cholesterol causes an inflammatory reaction (42).

Serum sTM was not different in any group. This is easily explained by the fact that after confirmation by an independent laboratory (Comparative Coagulation Section Diagnostic Laboratory, Cornell University, Ithaca, NY) it was found that the human sTM ELISA kit that I used did not cross-react with rabbit sTM.

Since I only measured one acute phase inflammatory marker, inflammation as the causitive factor of increased plaque development in the low Mg group cannot be ruled out. There are many inflammatory markers in the serum that could have been measured including IL-6, IL-1, and TNF-α (19). There are also more serum markers that directly relate to vascular endothelial injury that were not measured. These include: E-selectin; von Willebrand factor; ICAM-1; and VCAM-1, with von Willebrand factor (in addition to sTM) being the best candidate because it is strongly expressed upon endothelial damage (31). I hypothesize that the increase in atherosclerotic plaque development seen in the low Mg group was due to direct effects on the vascular endotheliam itself and that the serum markers that I used were not adequete to indicate this.

This study demonstrates that inadequete intake of Mg in rabbits results in an marked increase in atherosclerotic plaque development. CRP did not correlate with Mg intake

but rose in all groups, which supports the hypothesized basis of this animal model that large levels of cholesterol in the serum of rabbits is inflammatory. The mechanism by which adequete intake of Mg provided protection from atherosclerotic plaque develoment can not be discerned from the parameters measured in this study.

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